Tissue factor pathway inhibitor in patients with Diabetes Mellitus: an epiphenomenon?

TISSUE FACTOR PATHWAY INHIBITOR IN PATIENTS WITH DIABETES MELLITUS: AN *EPI*PHENOMENON ?

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TISSUE FACTOR PATHWAY INHIBITOR IN PATIENTS WITH DIABETES MELLITUS: AN *EPI*PHENOMENON ?

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Aan mijn vader
en mijn broer Rob

Contents

Chapter 1

Introduction

Introduction

It is well known that diabetes mellitus can lead to premature atherosclerosis and subsequent cardiovascular events. This is especially true for type 2 diabetes, where the co-existence of hyperglycemia, dyslipidemia and hypertension greatly increases cardiovascular risks. In type 1 diabetes the occurrence of microvascular complications like retinopathy and (incipient) nephropathy prevails. (Micro)-albuminuria thereby appears not only to be a marker of the development of nephropathy, but a strong predictor for cardiovascular disease as well[1-4].

Also from a quantitative point of view diabetes is a disease of great importance. Because of increasing age, altered life styles and the application of more stringent criteria for diagnosis, the number of patients is sharply rising worldwide[5]. This may explain the growing interest in preventive measures and interventions that could prevent the development of both type 1 (in ongoing studies like ENDIT using nicotinamide, or DPT-1 and EPP-SCIT using insulin[6,7]) and type 2 diabetes (by life style intervention and insulin-sensitizing drugs in DPP[8]). However, the results of these prevention trials will only be available in the next couple of years. Recently, the CAPPP and the HOPE study surprisingly suggested a decrease in the occurrence of (self-reported) diabetes, when patients with hypertension and/or cardiovascular disease were treated with an ACE inhibitor[9,10]. These interesting findings need further exploration and ascertainment.

Until the prevention of diabetes is a reality, we have to focus on the best possible treatment of the disease as well as on the prevention and treatment of its complications[11]. In the last decade it has been clearly demonstrated that improvement of metabolic control will result in less secondary (microvascular) complications in type 1 (Diabetes Control and Complication Trial (DCCT)[12,13]) as well as in type 2 diabetes (United Kingdom Prospective Diabetes Study (UKPDS)[14]). In addition, recent epidemiologic analysis of the UKPDS shows that macrovascular complications are also less prevalent with lower levels of glycated hemoglobin[15]. Endothelial damage precedes the development of atherosclerosis[16]. Increased levels of von Willebrand factor (vWF), thrombomodulin, tissue plasminogen activator (t-PA) and plasminogen activator inhibitor-1 (PAI-1) have been found in diabetic patients, especially in those with microvascular complications[17-19]. These alterations in endothelium-dependent hemostatic parameters may thereby reflect vascular endothelial damage. Most of these changes in levels are found when complications in the diabetic patient already exist. Until now, only vWF appears to precede the development of micro- and macroangiopathy in diabetes, and therefore may have a predictive value[20,21].

We hypothesized that due to its specific characteristics and important role in endothelium-derived initiation of coagulation, tissue factor pathway inhibitor (TFPI) may also be a marker of endothelial dysfunction and may even play a role in the

development of cardiovascular disease. This protein was identified in the late nineteen-eighties as a new Kunitz-type coagulation inhibitor[22]. TFPI contains 276 amino acid residues and, as a natural anticoagulant, it plays a key regulatory role in tissue factor-initiated blood coagulation.
Under normal physiologic conditions, TFPI is mainly synthesized by and bound to the vascular endothelial cells[23,24]. It can be released from the endothelial cells by heparin[25]. TFPI can also be found in platelets and in plasma, mostly associated with lipoproteins[26,27]. Only a small portion is found in a free uncomplexed form[28].
The studies represented in this thesis started late 1993. At that time, nothing was known about TFPI in diabetic subjects. These studies were specifically designed to evaluate not only the possible effect of diabetes on TFPI, but the possible role of TFPI in the development of vascular diabetic complications as well.

Aims of the thesis

In *Chapter 2*, we reviewed our present knowledge of TFPI in physiologic and pathophysiologic (non-diabetic) conditions. The studies described in the following chapters were undertaken to investigate the role of TFPI in diabetes. Firstly, we studied the effect of metabolic regulation on TFPI activity in type 1 diabetic patients without vascular complications, before and after intravenous administration of heparin (*Chapter 3*). Because TFPI activity may reflect endothelial damage, a second study was performed, in which we compared type 1 diabetic patients with and without microvascular complications (*Chapter 4*). As it has become clear that diabetes is characterized by the presence of a procoagulant state, TFPI activity could be influenced by this[29,30]. We therefore studied TFPI activity in type 1 diabetic patients and healthy volunteers in relation to coagulation using measurements of prothrombin F_{1+2} fragments and endogenous thrombin potential (*Chapter 5*). While type 1 diabetes is mainly complicated by microangiopathy, type 2 diabetes is mostly complicated by macroangiopathy. The condition of impaired glucose tolerance is considered to be a transitional phase to type 2 diabetes. We studied in a population-based study TFPI activity and other endothelium-dependent factors in eldery subjects with a normal or impaired glucose tolerance and type 2 diabetes mellitus (*Chapter 6*). To study the effects of typical diabetic conditions, e.g. hyperglycemia and hyperinsulinemia, on TFPI activity and release in more detail, cultured endothelial cells, derived from an immortalized endothelial cell line (EA.hy 926), were used (*Chapter 7*). Finally, the results are summarized and discussed in the general discussion.

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Chapter 2

Tissue Factor Pathway Inhibitor (TFPI) in physiologic and pathologic conditions

PB Leurs, R van Oerle, BHR Wolffenbuttel, K Hamulyák

Introduction

It was not until 1987 that an important protein was identified as a new Kunitz-type coagulation inhibitor[1]. It was a logical consequence of intense research in the years before, which focused on a factor, apparently circulating in the plasma, that exerted an inhibitory action on coagulation, initiated by the tissue factor pathway. This factor was first named anticonvertin[2]. Later the name varied from lipoprotein associated coagulation inhibitor (LACI) to extrinsic pathway inhibitor (EPI)[1,3,4]. Eventually, to unify the nomenclature, a concensus meeting in 1991 of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis agreed on the name tissue factor pathway inhibitor (TFPI).

The TFPI protein consists of 276 amino acid residues, with a molecular weight of 42 kDa, with a negatively charged acidic amino-terminal end, three tandemly repeated Kunitz type inhibitory domains and a positively charged basic carboxy-terminal end (Figure 2.1)[5]. More recently, a second human TFPI molecule, with 32 kDa of molecular weight containing 213 amino acids, has been isolated, which weakly inhibits factor Xa, but with a strong inhibitory effect on the factor VIIa/tissue factor complex[6].

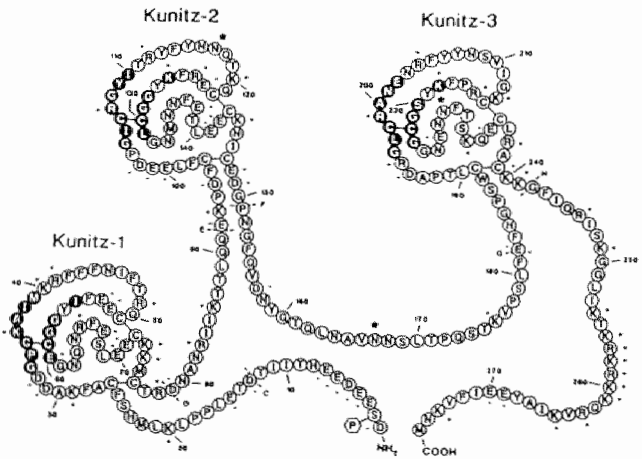

Figure 2.1 The TFPI protein contains 276 amino acid residues, with a negatively charged acidic amino-terminal end, three tandemly repeated Kunitz type inhibitory domains and a positively charged basic carboxy-terminal end. The 18 Cys residues in TFPI are involved in disulfide bonds.

The nonthrombogenic properties of the endothelial cell surface are maintained partly by protease inhibitors of the coagulation cascade. TFPI plays as a natural anticoagulant a key regulatory role in tissue factor-initiated blood coagulation.

Firstly, it directly inhibits factor Xa by a calcium-independent binding of the second Kunitz domain to factor Xa (Figure 2.2)[7]. Then, a calcium-dependent binding of the first Kunitz domain of the TFPI molecule to factor VIIa inhibits the factor VIIa/tissue factor catalytic complex. The formation of a quarternary complex occurs either via the binding of a TFPI/factor Xa complex to the factor VIIa/tissue factor complex or via binding of TFPI to the factor Xa/factor VIIa/ tissue factor complex[7-10]. Endothelial cell-associated TFPI may therefore play an important role in the anticoagulant property of endothelial cells.

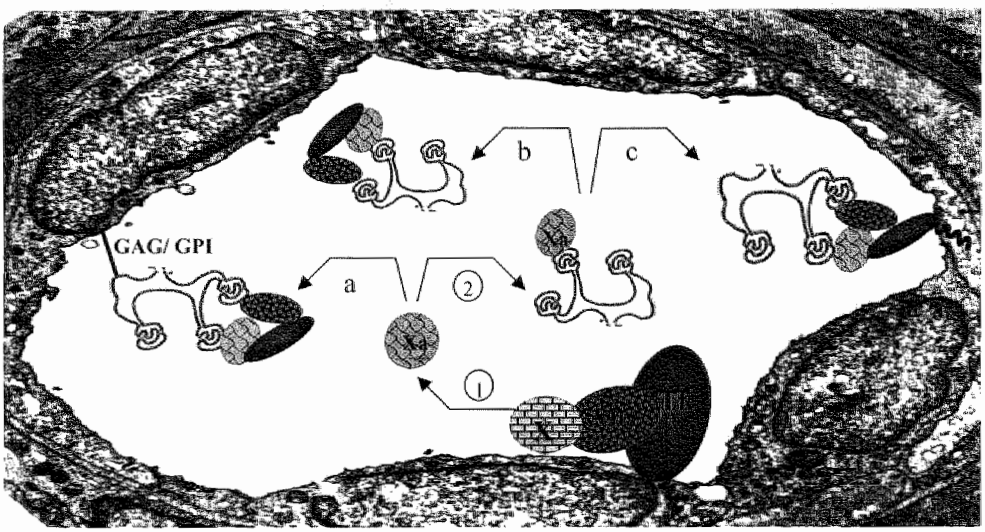

Figure 2.2 Anticoagulant properties of TFPI. After conversion of factor X to Xa by (transmembrane) glycoprotein tissue factor (TF) and factor VIIa (1), TFPI is able to inhibit this factor Xa by binding factor Xa to Kunitz domain 2 (2). This first step in the two-step reaction mechanism is Ca^{2+} independent. In the second step TFPI exerts it anti TF/VIIa activity by binding of the factor Xa/TFPI complex to the TF/VIIa complex. Hereby factor VIIa is bound to the Kunitz domain 1 of TFPI in a calcium-dependent way. Three major ways for formation of this quaternary complex can be distinguished [7-10]: TFPI/Xa complex is bound to TF/VIIa

a. whereby TFPI is bound to the endothelial cell surface by glycosaminoglycans (GAG) and/or glycosyl phosphatidylinositol (GPI).
b. free in plasma.
c. whereby TF is anchored in the plasma membrane.

Synthesis of TFPI

Under normal physiologic conditions, the synthesis and expression of TFPI is restricted to megakaryocytes and the endothelial cells of the microvasculature[11,12]. TFPI can also be found in macrophages in the villi of term placenta[11]. Increased levels of TFPI are commonly found in pregnancy[13]. More recently, TFPI expression has also been demonstrated in vascular smooth muscle cells[14,15].
The gene for TFPI is located on chromosome 2q and contains nine exons[16-18]. Exon 1 and 2 comprise the 5'untranslated region of TFPI mRNA. Exon 3 encodes for the signal peptide and NH_2-terminal head region of the TFPI protein. Exons 4, 6 and 8 encode for the Kunitz domains 1, 2, and 3, respectively. Exon 5 encodes for the linker region between domains 1 and 2, exon 7 encodes for the linker region between domains 2 and 3. Exon 9 encodes for the COOH-terminal tail region of the TFPI molecule. The TFPI gene containes GATA motifs which may regulate its expression by a GATA-2 transciption factor, which is produced by endothelial cells[19]. Recently, potential regulatory elements of the TFPI promotor region have been identified[20].
After synthesis, the TFPI proteins are stored in well-defined cytoplasmatic granules (caveolae), evenly spread over the cell surface of resting endothelial cells[21,22].

Binding of TFPI to endothelial cells

It is considered that TFPI molecules are exocytosed towards the surface of the endothelial cells, where it remains anchored probably to glycosaminoglycans, e.g. heparan sulfate[21,23-26]. The specific binding to glycosaminoglycans requires the highly positively charged carboxy-terminal end of the TFPI molecule, a binding of electrostatic nature[27]. It seems that TFPI is bound to the endothelial cell surface in a saturable manner[26,28]. Another region located in the third Kunitz domain binds to glycosaminoglycans like heparin with low-affinity[29]. However, not all studies indicate the involvement of glycosaminoglycans in the binding of TFPI to endothelial cells[30]. In addition to endothelial glycosaminoglycans, others have demonstrated glycosyl phosphatidylinositol (GPI)-anchored binding sites for TFPI molecules at the cell surface[22,31]. It is speculated that TFPI at GPI-anchored binding sites reflects heparin-unreleasable TFPI. On the other hand, TFPI bound to endothelial glycosaminoglycans, can be easily released by other glycosaminoglycans such as heparin[23]. Repeated intravenous injections and continuous intravenous infusion of heparin leads to a considerable depletion of heparin releasable TFPI[28]. Depending on the assay used, the release of TFPI after heparin results in a 2-4 fold increase in TFPI activity[23,32], 3-10 fold increase in total TFPI antigen levels[32,33], and 8-15 fold increase in free TFPI antigen levels[28].

TFPI in plasma

TFPI, bound to the endothelial cell surface, comprises the major pool (50-80%) of 3 intravascular pools, in which TFPI can be found. In plasma, the second pool, 10-50% of TFPI is located, while platelets serve as the third pool, containing only a fraction of total TFPI[34]. The physiological plasma concentration of TFPI is about 100 ng/ml or 2.5 nmol/l, but is increasing with age[33,35]. Most of TFPI in plasma is associated with lipoproteins LDL, HDL and Lp(a)[36-38]. The major form of TFPI associated with LDL is 34 kDa, whereas that associated with HDL is about 41 kDa. It is suggested that the carboxy-terminal end of TFPI is involved in the interaction between TFPI and lipoproteins[39]. However, this is contradicted by the finding that TFPI, bound to LDL, lacks a substantial portion of the carboxy-terminal end, including at least a portion of the third Kunitz domain[38]. The C-terminus of TFPI is essential for its anticoagulant activity[40,41]. It is therefore not surprising that only a weak anticoagulant activity of lipoprotein associated TFPI can be demonstrated[33,42-44]. On the other hand, free uncomplexed TFPI, which consists 5-10% of total TFPI in plasma, exerts a strong anticoagulant activity[33,43]. This free portion of TFPI is inversely correlated with HDL[45].
The heparin-releasable TFPI is almost entirely carrier-free and it is known to contribute significantly to the anticoagulant action of heparins[43,46,47].

Degradation and clearance of TFPI

Until now, little is known about the degradation and clearance of TFPI in humans. From pharmacokinetic studies in rabbits it is known that the clearance of recombinant TFPI exhibited a bi-exponential elimination with a rapid α phase half-life of 2.3 min, due to a rapid redistribution and tissue uptake, especially in the liver and kidney (outer cortex), and a terminal ß phase half-life of 79 min[48]. The half-life of full-length TFPI is markedly shorter than of TFPI truncated at the carboxy-terminus[49]. The rapid hepatic and renal clearance of TFPI stimulated others to look for specific receptors, which mediated the uptake into tissues. It was found that in rat and human hepatoma cell lines TFPI cellular degradation, but not binding to the cell, is mediated by low density lipoprotein receptor-related protein (LRP), a cell-surface glycoprotein that functions as a hepatic endocytosis receptor for several plasma proteins[50,51]. It appears that the LRP-mediated clearance of TFPI occurs in two steps. In the initial step, which requires the presence of the C-terminus and can be inhibited by heparin, TFPI binds to an unrelated cell-surface receptor, possibly glycosaminoglycans, before it is presented to LRP for uptake and degradation, presumably by endocytosis[50-52]. In contrast, others could not demonstrate a degradation of exogenously added TFPI by human umbilical vein

endothelial cells (HUVEC), although it was bound to the cell surface[53]. Because of the presence of endogenous TFPI in endocytic and recycling compartments of HUVEC, it is hypothesized that endogenous, membrane-anchored TFPI could be internalized for subsequent recycling back to the cell surface[53].

Cell surface glycosaminoglycans like heparan sulfate associated with endothelial cells have been proposed to play a role in the clearance of TFPI[23]. More recently, a study in mice suggested the involvement of two independent receptor systems in the clearance of TFPI, i.e. LRP and heparan sulfate proteoglycan[54]. Underlining the role of glycosaminoglycans in the clearance of TFPI, it appears that heparan sulfate proteoglycans are required for uptake and degradation of TFPI-factor Xa complexes, while factor Xa binding stimulates the internalization and degradation of cell surface-bound TFPI[10,55]. Another study also provided evidence for a dual role of factor Xa[56]. Besides its role as an essential cofactor in the TFPI-controlled regulation of tissue factor-dependent coagulation, it was shown that factor Xa cleavage of TFPI is associated with loss of anticoagulant activity. A novel degradation pathway of TFPI is demonstrated by Ohkura et al.[57] Hereby, TFPI is incorporated into fibrin clots via interaction of basic amino acid residues in the C-terminal region of TFPI with negatively charged residues in fibrin. Thereafter, TFPI is degraded by fibrin-bound thrombin, probably by cleaving peptide bonds in the TFPI molecule.

More recently, proteolysis of rTFPI by plasmin is demonstrated, suggesting that TFPI is susceptible to proteolysis when the plasma fibrinolytic system is activated[58].

TFPI in pathologic conditions

When the tissue factor pathway is activated by exposure of blood to tissue factor at an injury site the coagulation cascade is initiated by the formation of the factor VIIa-tissue factor catalytic complex. This complex has the ability to activate factor IX into IXa and factor X into Xa by proteolysis[59]. This eventually leads to the generation of thrombin and the formation of a fibrin clot. In addition, endothelial cells and monocytes are the predominant cells that may express tissue factor during an inflammatory disease. It is demonstrated that HUVEC are capable to express tissue factor in reponse to various stimuli, known to be important during inflammation[60-63]. However, while expression of tissue factor was markedly increased in response to inflammatory mediators like endotoxin, interleukin-1 and tumor necrosis factor-α, TFPI secretion into the media was unchanged or only slightly higher in induced HUVEC cultures[64]. These data indicate that TFPI synthesis is not downregulated, but may be slightly upregulated during an infammatory response[64]. Factor Xa binding stimulates the uptake and degradation

of cell surface-bound TFPI[10]. On the other hand, it has recently been shown that upon treatment of cultured endothelial cells with thrombin, a marked redistribution and acute release of TFPI occurred from specific granules[21].

Disseminated intravascular coagulation (DIC) appears to be related to vascular endothelial cell injury because plasma levels of endothelial-dependent hemostatic parameters, i.e. thrombomodulin, tissue type plasminogen activator (t-PA), plasminogen activator inhibitor-1 (PAI-1), and von Willebrand factor (vWF) are shown to be increased in DIC[65,66]. Tissue factor is a major initiator of the onset of DIC[67,68]. It appeared that TFPI and tissue factor both were increased in DIC, but tissue factor did not correlate with TFPI[69]. More recently, it is demonstrated that TFPI, following an increase in tissue factor, is increased in patients with DIC, suggesting that TFPI may reflect vascular endothelial damage[70]. An earlier report suggested no increase of TFPI in DIC in patients with hepatocellular disease[71]. Lower TFPI levels have been found in cirrhotic patients[12,72]. It is thereby postulated that the decrease in TFPI may be due to decreased levels of LDL cholesterol secondary to hepatocellular dysfunction.

Systemic coagulation activation is a well-recognized feature of malignancy, partly because tumor cells produce factors which directly and indirectly could induce coagulation activation[73-75]. Increased TFPI activity is also found in cancer patients[42]. It is postulated that this increase could result from the production of host cells, possibly endothelial cells stimulated by cytokines from the malignant process, or from products of hypercoagulation[42]. Later, others demonstrated that tumor-associated macrophages in several types of malignancy, which are known to express a complete tissue factor-initiated pathway of coagulation and thrombin generation, also express TFPI[11]. By contrast, malignant cells, known to express coagulation factors together with tumor cell-associated fibrin formation failed to stain immunohistochemically for TFPI[11]. These results may indicate that TFPI may be lacking from the latter malignancies because of the absence of tissue factor/factor VIIa-factor/Xa complex, required for TFPI binding.

Decreased plasma TFPI activity have been found in patients with ischemic stroke, while no relationship could be established between TFPI activity and other haemostatic parameters reflecting procoaculant activity[76]. Therefore, the moderately lower TFPI activity in stroke patients could be due to atherosclerotic changes rather than to consumptive coagulopathy[76]. In contrast, elevated plasma TFPI activity has been demonstrated in patients with acute coronary disease, in whom a moderate increase in procoagulant activity (factor VII-phospholipid complex) was also seen, suggesting that TFPI adapts to procoagulant changes[77]. However, no relation between factor VII and TFPI activity was found by others, but, instead, a close association between TFPI and plasma levels of dense LDL and small, dense HDL particles was shown[78]. Recently, Petit et al. showed in an in vitro experiment that the procoagulant activity of tissue factor in macrophage-derived foam cells is not

counterbalanced by upregulation of TFPI activity[79]. Some have suggested that the increased TFPI after acute myocardial infarction might be released from ischemic tissue[80]. The observation of a severe depletion of heparin-releasable TFPI in patients undergoing percutaneous transluminal coronary angioplasty could be of importance, considering the acute thrombotic occlusion which may occur following this procedure[81]. This observation was later confirmed by a study, in which depletion of intravascular pools of TFPI was demonstrated during continuous intravenous infusion of heparin in healthy volunteers[28]. Also in patients suffering from ischemic heart disease like unstable or effort angina elevated TFPI levels have been observed[82,83]. It was thereby suggested that the blood clotting activation in these patients may be related to elevated tissue factor circulating levels not sufficiently inhibited by the elevated TFPI plasma levels. Recently, it is suggested that TFPI exon IX polymorphism may be associated with subtypes of myocardial infarctions[84]. On the other hand, TFPI has been demonstrated in human carotid atherosclerotic plaques, which was associated with a reduced tissue factor activity within the plaque[85]. Others found increased heparin-releasable TFPI levels in the coronary circulation after coronary spasm in patients with coronary spastic angina[86]. Recently, increased expression of TFPI in atherosclerotic vessels has been demonstrated[87].

In dyslipemic patients, TFPI activity may vary, depending on the variations of its main lipoprotein carriers[88]. In general, TFPI activity is increased in hypercholesterolemic patients[89], although decreased levels of carrier-free TFPI in hyperlipidemic subjects have also been found[90]. Treatment of patients with familial hypercholesterolemia with hydroxymethylglutaryl-coenzyme A reductase inhibitors resulted in a marked decrease of TFPI activity, with a close correlation with corresponding alterations in LDL levels[89]. It appears that the effect of cholesterol lowering on the intravascular pools of TFPI does not affect the size or the anticoagulant potency of the endothelial pool of TFPI[43]. A daily dosage of 3 g n-3 polyunsaturated fatty acids in patients with chronic atherosclerotic disease resulted in a significant but small increase of TFPI plasma levels, which was correlated with increased LDL levels[91]. Simultaneously, a significant reduction of F_{1+2} levels with no changes in plasma factor VII clotting activity was shown, indicating a down-regulation of the extrinsic pathway of blood coagulation. However, others did not find any effect of n-3 fatty acids on plasma factor VII and TFPI[92]. Monounsaturated-rich diet has been demonstrated to induce a decrease in plasma levels of TFPI and other endothelium-derived factors in young healthy males, suggesting a beneficial effect on endothelial function[93]. In plasma of hypercholesterolemic monkeys on a high-cholesterol diet an increase in LDL/VLDL-associated TFPI was seen, while there was no difference in postheparin TFPI between normal and hypercholesterolemic animals[94]. It was suggested that the results reflect a decrease of TFPI on endothelial cells in

atherosclerotic lesions.
It is becoming increasingly clear that the oxidative modification of LDL cholesterol underlies the atherogenicity of these cholesterol-rich particles[95,96]. Recently, it is demonstrated that TFPI activity, associated with LDL is inactivated by cell- and copper mediated oxidation[97]. This oxidative inactivation of LDL-associated TFPI may therefore effectively neutralize its inhibitory action on tissue factor activity, resulting in a disequilibrium in favor of coagulation, especially in the atheromatous plaque, where tissue factor is overexpressed.
Finally, there are two other groups of patients, in which altered TFPI levels may suggest vascular endothelial damage, i.e. patients with thrombotic thrombocytopenic purpura and in hemodialysis patients[98-100]. In addition, high levels of TFPI were found in patients with nephrotic proteinuria[101]. In renal biopsies of patients with crescentic glomerulonephritis, the expression of TFPI was increased, suggesting that TFPI may inhibit tissue factor activity and favor reduced fibrin deposition in the chronic stages of crescent formation[102]. Also in adult respiratory distress syndrome and interstitial lung disease increased levels of TFPI are reported, possibly due to synthesis of TFPI by the lung epithelial lining cells and/or endothelial cells of the microvasculature[103,104].
Recently, in women taking combined oral contraceptives a decrease in plasma TFPI has been demonstrated, suggesting a possible explanation for the thrombotic effect of oral contraceptives[105].

Potential therapeutic applications of TFPI

With the development of recombinant TFPI (rTFPI) it became possible to explore the usefulness of TFPI as a therapeutic agent. In animal studies, using rabbits, it was shown that rTFPI ameliorated the consequences of DIC, induced by tissue thromboplastin or endotoxin[106,107]. In baboons, rTFPI reduced the mortality of septic shock caused by Escherichia coli, while in pigs TFPI treatment only resulted in an attenuation of mediators of the inflammatory response, but did not provide survival benefit[108,109].
Recombinant TFPI is also used to prevent thrombosis in conditions, in which a beneficial effect of rTFPI can be expected because of exposure to tissue factor[110]. Increased levels of TFPI are found in healthy subjects, using elastic stockings during upright passive tilting[111]. It is thereby suggested that TFPI may represent a possible mechanism by which prophylaxis of deep vein thrombosis with the use of elastic stockings can be explained. In vein bypass grafting procedures it appeared that rTFPI is able to prevent thromosis effectively[112]. Besides, rTFPI is shown to prevent restenosis after thrombolysis and balloon-induced arterial injury, possibly by attenuation of procoagulant activity and tissue factor-mediated thrombin

generation in response to injury[113-116]. In vitro, rTFPI can also reduce the thrombogenicity of disrupted human atherosclerotic plaques by inhibition of tissue factor[117]. Recombinant TFPI is probably also an effective agent in limiting postoperative paraplegia associated with spinal ischemia combined with ischemia/reperfusion injury in rabbits[118]. In rats, rTFPI strongly inhibits the injury of ischemic reperfusion of the liver after clamping the hepatic artery and portal vein for 2 to 3 hours[119]. In vivo TFPI gene transfer into an injured artery completely inhibits the recurrent thrombosis induced by shear stress without affecting the systemic coagulation status in rabbits and pigs[120,121].

In rats, exogenous TFPI can increase anticoagulant activity on the hepatic sinusoidal walls by binding to heparinoids on the cell surface[122].

In man, no studies have yet been performed with rTFPI, but the animal studies thusfar are promising for rTFPI as a therapeutic mean in the near future.

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Chapter 3

Tissue Factor Pathway Inhibitor (TFPI) activity in patients with IDDM

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Abstract

Until now, several endothelium-dependent hemostatic parameters have been proposed as markers of vascular endothelial dysfunction in diabetes. We studied tissue factor pathway inhibitor (TFPI) activity in IDDM patients without macro- and microvascular complications, before and after intravenous administration of heparin, in comparison with age-matched controls. We also examined the effect of acute hyperglycemia on TFPI activity in healthy men. A clotting and chromogenic assay were used for determining TFPI activity. In the clotting assay the C-terminus of TFPI is essential, while this region is of minor importance in the chromogenic assay. When the chromogenic assay was used, TFPI activity before heparin injection was significantly higher in the IDDM patients (92 ± 24 vs $112 \pm 23\%$, $p < 0.01$). The postheparin increase in TFPI activity, measured with both assays, was significantly higher in the diabetic subjects (area under the curve: clotting assay 64 ± 14 vs 81 ± 24, $p < 0.05$; chromogenic assay 82 ± 26 vs 121 ± 35, $p < 0.0001$). A positive correlation between TFPI activity and glycated hemoglobin was demonstrated. Acute hyperglycemia did not alter TFPI activity. It is concluded that TFPI activity, especially after stimulation with heparin, is affected by chronic hyperglycemia in diabetic subjects without vascular complications. Alterations in TFPI activity may therefore reflect early endothelial dysfunction.

Introduction

Diabetes mellitus is associated with premature atherosclerosis. It is known that endothelial cells play an important role in the repair process of vascular injury, thereby maintaining the functional integrity of the vascular wall. Endothelial damage has been considered as one of the initiating events in the pathogenesis of atherosclerosis[1].

The vascular endothelium can be regarded as a paracrine organ which regulates hemostasis (coagulation and fibrinolysis). It produces procoagulant substances (e.g. von Willebrand factor, tissue factor) and components of the fibrinolytic system that promote or inhibit fibrinolysis (tissue plasminogen activator (t-PA) and plasminogen activator inhibitor-1 (PAI-1), respectively)[2,3]. Endothelium also expresses factors associated with anticoagulant properties such as heparan sulphate and thrombomodulin[2,3]. In 1987 a new Kunitz-type coagulation inhibitor, tissue factor pathway inhibitor (TFPI), was identified as a single protein[4]. Coagulation activity, initiated by the tissue factor pathway, is activated by the complex of tissue factor and factor VIIa. This complex not only activates factor X but factor IX via the Josso loop as well[5,6]. TFPI directly inhibits factor Xa and in complex with factor Xa also the tissue factor/factor VIIa catalytic complex[7]. The major site of production of TFPI is the vascular endothelium[8]. Of TFPI, 50-90% is found on the endothelium, 10-50% in plasma and the remainder in platelets[9]. Most of TFPI in the plasma is associated with lipoproteins[10,11]. Only about 5% of the TFPI in the plasma circulates as a free, uncomplexed protein[9]. TFPI activity is altered in several pathological conditions[12-14]. Its release from the endothelium can be stimulated by heparin[15].

In diabetic patients with microvascular complications increased levels of von Willebrand factor, thrombomodulin, t-PA and PAI-1 are found[16-18]. It is therefore suggested that these alterations in endothelium-dependent hemostatic parameters reflect vascular endothelial damage. Little is known about TFPI activity in diabetic subjects. Because TFPI may also be regarded as a marker of endothelial dysfunction, we studied TFPI activity in IDDM patients without vascular complications before and after intravenous administration of heparin. The effect of acute hyperglycemia on TFPI activity was investigated in healthy men. We also examined the relationship between the degree of metabolic regulation and TFPI activity.

Materials and Methods

Twenty-five IDDM patients (12 men, 13 women; mean age 33 (range 19-49) years) and 21 age-matched healthy controls (10 men, 11 women; mean age 33 (range

22-43) years) were studied. Only IDDM subjects without neuropathy, retinopathy, nephropathy and macrovascular complications were included. Thus, all patients were normoalbuminuric and normotensive. Patients were also divided according to the degree of metabolic control. The characteristics of the subjects are depicted in Table 3.1.

Table 3.1 Demographic and laboratory parameters of IDDM patients and controls at baseline.

	Controls	IDDM		
		Overall	HbA_{1c} <7.4%	HbA_{1c} ≥8.4%
	I	II	III	IV
n	21	25	12	13
Sex ratio	10M/11F	12M/13F	6M/6F	6M/7F
Age (years)	33±6	33±8	35±7	32±8
Duration diabetes (years)		9 (1-31)	9 (1-31)	9 (1-21)
Dosage insulin (IU/day)		48±17	43±15	53±16
Body mass index (kg/m^2)	23.7 ± 2.1	23.3±2.5	22.7±2.8	23.8±2.0
Glycated haemoglobin (%)	4.9 ± 0.4*	8.1±1.6	6.7±0.5§	9.5±0.9
Total cholesterol (mmol/L)	5.0 ± 0.7	5.3±1.4	4.9±0.6	5.8±1.7
Triglycerides (mmol/L)	0.87 (0.24-2.08)	1.12 (0.5-3.56)	0.98 (0.5-3.56)	1.12 (0.76-1.66)
HDL-cholesterol (mmol/L)	1.34 ± 0.33	1.43±0.42	1.41±0.45	1.46±0.40
LDL-cholesterol (mmol/L)	3.21 ± 0.63	3.32±1.46	2.81±0.67	3.78±1.78
Apo A_1 (g/L)	1.51 ± 0.18	1.60±0.22	1.60±0.25	1.61±0.21
Apo B (g/L)	0.93 ± 0.22	1.05±0.33	0.94±0.17	1.14±0.39
Lipoprotein (a) (U/L)	139 (17-1478)	126 (12-1714)	126 (21-848)	143 (12-1714)
Serum insulin (pmol/L)	41.4 (19.8-69)	49.2 (18-189)	45 (22.2-189)	49.2 (18-149.4)

Data shown as mean ±SD, or median (range). * = $p<0.05$ vs II, III and IV; § = $p<0.05$ vs IV

Each study started at 08.00 a.m. while the subject was still fasting. With the subject in supine position bloodsamples were taken via an indwelling catheter in a forearm vein at 15 and 0 minutes before and 3,5,10 and 30 minutes after an intravenous bolus injection of 5000 IU of unfractionated heparin (Leo, Denmark) in the contralateral forearm. For determining TFPI activity, von Willebrand factor, thrombomodulin, t-PA and PAI-1, bloodsamples were collected in tubes containing sodium citrate 3.25% (dilution 1:10). Bloodsamples were also drawn for determination of glycated hemoglobin and lipid profile at baseline. Glycated hemoglobin (HbA_{1c} fraction) was measured immediately by a HPLC-method (Diamat, Bio-Rad Laboratories, USA), the remainder of the bloodsamples were centrifuged and plasma was stored at -70°C until further processing.

A second study regarding the effect of an acute glucose load on TFPI activity was performed in 6 healthy men (mean age 30 (range 24-36) years; mean BMI 23.1±1.4 kg/m^2). After an overnight fast, with the subject in supine position, bloodsamples were taken before and 10 and 30 minutes after intravenous administration of glucose 50% (0.5 g/kg body weight with a maximum of 35 g) in three minutes time in the contralateral forearm. Bloodsamples for glucose, insulin and C-peptide determinations were also taken. Both protocols were approved by the Ethical Committee of the University Hospital. Written informed consent was obtained from all participants.

TFPI activity was measured using both a modified clotting assay based on the diluted tissue thromboplastin clotting time as reported previously (expressed in seconds; CV 4.5%)[19] and the chromogenic substrate assay according to Sandset (expressed in % with regard to standardized TFPI activity, measured in a plasma pool obtained from 45 healthy donors; CV 7.4% at 100% level)[20]. The tissue factor concentration, used in the clotting assay, was adjusted to a coagulation time of 123 seconds measured in normal plasma[19]. With this tissue factor concentration, an optimal balance between accuracy and sensitivity of the clotting assay was achieved. Two different assays were used because with the clotting method mainly full-length TFPI, which has a very strong bioactivity, is measured, while the chromogenic method also measures C-terminal fragmented TFPI, which has only a weak anticoagulant activity. The area under the curve (AUC) for TFPI activity was also calculated. Plasma levels of von Willebrand factor were determined by an ELISA method using rabbit anti-human von Willebrand factor (Dako A/S, Denmark; CV 7%; normal range 60-180%). Because of the well-known relation between von Willebrand factor and blood group O[21], the blood of the study population was typed. Thrombomodulin determination was performed with an enzyme immunoassay (Diagnostica Stago, France; CV 8-10%; normal range 50-120 ng/ml, mean 76±22 ng/ml). An enzyme immunoassay was also used for the measurement of t-PA antigen (Innogenetics, Belgium; CV 7%; normal range 1.3-10.4 ng/ml, mean 4.1±2.4 ng/ml). PAI-1 activity was photometrically measured (Kabi Diagnostica, Sweden; intra-assay CV 0.4-2.4%; normal range 1-20 AU/ml, median 8 AU/ml). Total and HDL-cholesterol and triglycerides were determined with enzymatic colorimetric tests (Unimate 5 and 7, Roche, Basle, Switzerland), and apo A_1 and apo B were measured with immunoturbidimetry (Uni-kit, Roche). LDL-cholesterol was calculated with the Friedewald formula. Lipoprotein (a) was determined by a solid-phase, two-site immunoradiometric assay using two monoclonal antibodies directed towards different epitopes of apo(a) (Pharmacia Diagnostics, Uppsala, Sweden). At a Lp(a) concentration of 200 U/L the within-assay coefficient of variation was 4%. An enzymatic hexokinase method (Unimate 5, Roche) was used for measuring serum glucose concentrations. Free serum insulin was measured with a double antibody radioimmunoassay (Pharmacia Diagnostics;

within-assay CV 3.4-6.1% in the range of 18-300 pmol/L) after polyethylene glycol pretreatment. C-peptide concentration in serum was also determined by RIA (Byk-Sangtec Diagnostica, Germany; within-assay CV 1.9-5.0% in the range of 0.10-1.75 nmol/L).

Statistics

All data are expressed as mean±SD or as median and range when not normally distributed. Differences between two group means were tested with a *t*-test. When data were not normally distributed the Mann-Whitney-Wilcoxon rank-sum test was applied. To analyse postheparin TFPI changes a MANOVA for repeated measurements was performed. For comparing more than two groups, one-way analysis of variance with Student-Newman-Keuls correction for multiple comparisons was used. The Kruskal-Wallis analysis of variance was applied if data were not normally distributed.
P-values ≤ 0.05 were considered statistically significant.

Results

The IDDM patients and controls were well matched regarding demographic parameters (Table 3.1). There were also no significant differences between the well and poorly- controlled diabetic subjects with respect to duration of diabetes and daily insulin dosage. Lipid profile, Lp(a), apo A_1, apo B and insulin concentrations were similar in the diabetic and control group. There was also no significant correlation between these (apo)lipoproteins or serum insulin levels and the endothelium-dependent indices. Regarding the endothelium-dependent coagulant and fibrinolytic proteins at baseline, plasma levels of thrombomodulin and PAI-1 activity were not different between both study groups (Table 3.2). In contrast, t-PA antigen was significantly lower ($p<0.01$) in the diabetic subjects compared to the controls, independently of the degree of metabolic control, while the plasma level of von Willebrand factor was higher ($p<0.01$). However, levels of t-PA antigen and von Willebrand factor in the diabetic patients were still within normal ranges. A significant correlation between t-PA and TFPI activity, when measured with the clotting assay, was found ($r=0.56$, $p<0.01$). There was no difference in blood groups between the control and diabetic subjects. TFPI activity before administration of heparin was similar in the various groups when it was measured with the clotting assay.

Table 3.2 Hemostatic parameters of IDDM and healthy subjects at baseline.

	Controls	IDDM		
		Overall	HbA1c <7.4%	HbA1c ≥8.4%
	I	II	III	IV
Von Willebrand factor (%)	84.7±24.3*§	103.5±21.8	109.0 ± 22.3	98.5 ± 21.0
Thrombomodulin (ng/ml)	80.1±22.7	80.6±22.3	85.2 ± 20.5	76.5 ± 23.9
t-PA (ng/ml)	8.77±2.61*‖	6.65±2.23	6.82 ± 2.42	6.49 ± 2.11
PAI-1 (AU/ml)	6.9 (1.0-25.9)	4.3 (1.0-16.0)	5.0 (1.0-15.9)	2.9 (1.0-16.0)
TFPI activity (sec)[a]	116±10	116±11	115 ± 10	117 ± 11
TFPI activity (%)[b]	92±24*	112±23	101 ± 12	122 ± 25¶

Data shown as mean ±SD, or median (range). a= clotting assay, b= chromogenic assay. *= p<0.01 vs II; §= p<0.05 vs III; ‖= p<0.05 vs III and IV; ¶= p<0.05 vs I and III

In contrast, when measured chromogenically, TFPI activity was significantly higher (p<0.01) in the diabetic group at baseline. In poorly controlled patients, TFPI activity was higher (p<0.05) than in well-controlled subjects (Figure 3.1).

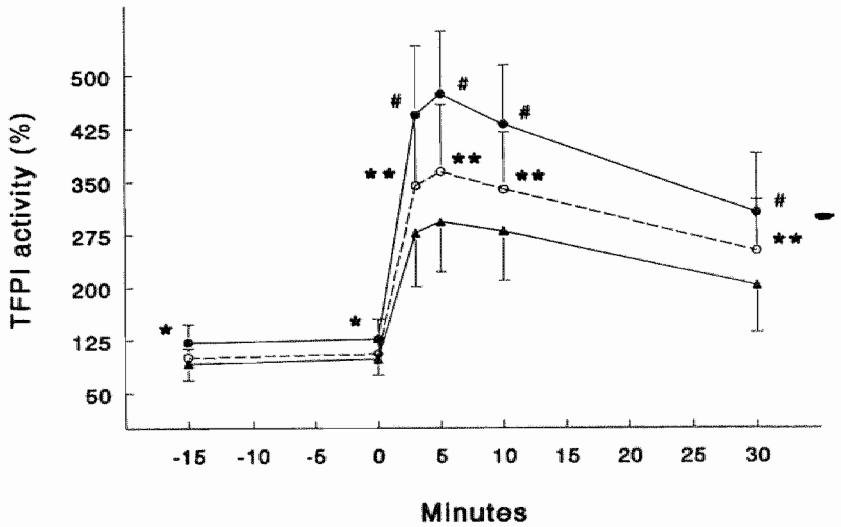

Figure 3.1 The increment of TFPI activity, measured chromogenically, after i.v. bolus injection of 5000 IU of heparin in well (○; n=12) and poorly (●; n=13) regulated IDDM patients and healthy controls (▲; n=21). Before administration of heparin, TFPI activity is significantly higher in the poorly regulated patients (* = p<0.05 vs well regulated group, p<0.01 vs controls). The difference in increase in postheparin activity is already significant 3 minutes after stimulation (# = p<0.05 vs well regulated group, p<0.0001 vs controls; ** = p<0.05 vs controls).

The postheparin TFPI activity in the diabetic subjects was higher than in the controls. The difference in increase of TFPI activity, measured chromogenically, was significant at all time points after intravenous administration of heparin ($p<0.0001$). When diabetes control was poor, the postheparin increase of TFPI activity was significantly higher than in case of a good metabolic regulation (at 5 min $264\pm95\%$ vs $352\pm73\%$, $p<0.05$; Figure 3.1). The maximum peak in TFPI activity in the IDDM group ($421\pm106\%$) and controls ($293\pm71\%$) was seen 5 minutes after heparin administration. A significant correlation was found between HbA_{1c} and the AUC of postheparin TFPI activity ($r=0.68$, $p<0.001$; Figure 3.2). When applying the clotting assay, comparable results were seen. The increase in TFPI activity was significantly higher in the diabetic subjects at 5 to 30 minutes postheparin ($p<0.05$). There also existed a correlation between the AUC of postheparin TFPI activity and HbA_{1c} ($r=0.45$, $p<0.001$).

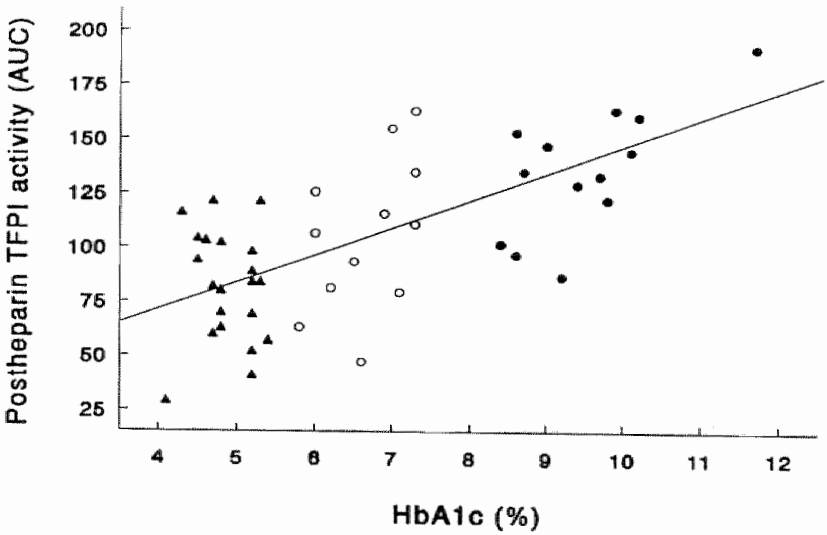

Figure 3.2 A significant correlation ($r=0.68$, $p<0.001$) is found between the degree of metabolic regulation and the AUC of the chromogenically measured postheparin TFPI activity. When the clotting assay was used, a weaker correlation was found ($r=0.45$, $p<0.001$). ▲: healthy controls, ○: well regulated, ●: poorly regulated IDDM subjects.

In the second study the mean fasting glucose concentration was 4.2 ± 0.2 mmol/L which increased to a maximum of 16.1 ± 1.4 mmol/L one minute after intravenous administration of glucose. After 10 and 30 minutes the glucose levels were 11.9 ± 0.8 and 7.2 ± 1.9 mmol/L, respectively (Table 3.3). TFPI activity, measured with both assays, did not change after induction of acute hyperglycemia.

Table 3.3 Effect of an acute glucose load on TFPI activity in 6 healthy males.

	0 min	10 min	30 min
Glucose (mmol/L)	4.2±0.3[*]	11.9±0.8[§]	7.2±1.9
Insulin (pmol/L)	29.4 (9.6-65.4)[‖]	132 (61.6-316.2)	82.8 (58.2-228)
C-peptide (nmol/L)	0.77±0.29[¶#]	2.20±0.77	2.04±0.70
TFPI activity (sec)[a]	125±6	128±4	127±7
TFPI activity (%)[b]	115±18	106±18	106±21

Data shown as mean ±SD, or median (range). a= clotting assay, b= chromogenic assay. [*]= $p<0.0001$ vs 10 min and 30 min; [§]= $p<0.0001$ vs 30 min; [‖]= $p<0.01$ vs 10 min and 30 min; [¶]= $p<0.001$ vs 10 min; [#]= $p<0.005$ vs 30 min

Discussion

TFPI is regarded as an important protein in the regulation of hemostasis. Besides, TFPI activity may reflect endothelial function because TFPI is produced by and mainly found on vascular endothelium. It is possibly bound to the endothelial cell surface by glycosaminoglycans[9]. The C-terminus of TFPI, which is important for binding glycosaminoglycans like heparin, can be truncated by various enzymes, resulting in considerable loss of its anticoagulant activity[22,23]. In the present study two different assays for determining TFPI activity were used. The modified clotting assay measures the anticoagulant full-length TFPI, while the chromogenic assay also measures C-terminal truncated TFPI. In comparison with healthy controls, a significantly higher increase in TFPI activity after stimulation with heparin was observed in our IDDM subjects. This was true for both assays. When applying the clotting assay, the increase in TFPI activity appeared to be independent of the activity at baseline.

It is well known that in diabetic subjects several hemostatic parameters are disturbed. These include coagulant and fibrinolytic components that, just as TFPI, are dependent on the vascular endothelial cell function[24,25]. Our results indicate that TFPI activity after stimulation with heparin is influenced by the chronic hyperglycemic condition. Not only a higher increase in TFPI activity in the diabetic patients was seen but also a significant positive correlation between postheparin TFPI activity and HbA_{1c} was found. No relation between TFPI activity and serum insulin levels could be demonstrated. There was also no effect of acute hyperglycemia on basal TFPI activity in healthy subjects.

It is known that TFPI has an association with the lipoproteins LDL, HDL and Lp(a)[10,11]. In the present study, mean total and LDL-cholesterol and the median of Lp(a) levels tended to be higher in poorly-regulated diabetic patients than in the well-regulated diabetic and control group, but this difference did not reach

statistical significance. We did not find any correlation between TFPI activity at baseline or postheparin and plasma (apo)lipoproteins.

An important feature of diabetes mellitus is the occurrence of non-enzymatic glycation reactions in which also proteins, important in hemostasis, are involved[26]. The difference in the measurements of TFPI activity between the clotting and chromogenic method, especially at baseline, could be the result of a difference in sensitivity between both assays. However, it may also suggest the presence of fragmented TFPI in diabetic patients. This may be the result of non-enzymatic glycation. Besides, it is thought that TFPI is bound to the endothelial cell surface by glycosaminoglycans[9]. Heparin is able to release proteins that are bound to glycosaminoglycans, such as platelet factor 4 (PF4), from the endothelium[27]. In diabetic subjects, the release of PF4 after intravenous administration of heparin was significantly higher than in healthy controls[27]. Heparin probably also binds to lysine residues as in antithrombin III, resulting in a more rapid reaction with coagulation proteases[28]. It has been demonstrated that this heparin cofactor activity of antithrombin III is altered by non-enzymatic glycation[26]. As already mentioned, the C-terminus of TFPI, which contains lysine residues, is essential for binding to heparin. We speculate that in diabetes the C-terminus of TFPI is affected by the process of non-enzymatic glycation. This may lead to functional alterations of these molecules resulting in an altered binding between glycosaminoglycans and TFPI, which may be responsible for the excess of release of TFPI in diabetic patients after injection of heparin. An additional explanation may be the non-enzymatic glycation of apolipoproteins A_1 and B[29,30]. The C-terminus of TFPI thereby appears to be involved in the interaction between TFPI and the lipoproteins[31]. It has been shown that glycation diminishes the interaction between apolipoprotein A_1 and the HDL-particle[30]. If one presumes that this also occurs between the lipoproteins and TFPI, one may expect a redistribution of TFPI in favour of the vascular endothelium. In that case, a higher response in TFPI activity after heparin injection may occur.

Non-enzymatic glycation eventually results in the formation and accumulation of advanced glycation endproducts (AGEs). AGEs are able to affect the endothelial cellular function by interacting with specific receptors, inducing an increase in tissue factor activity and a reduction in thrombomodulin activity[32]. The altered TFPI activity in diabetes may also be related to functional alterations in the endothelium by AGEs.

Increased oxidative stress as seen in diabetes is accompanied by abnormalities in hemostatic parameters, especially in subjects with microalbuminuria[18,33]. Collier et al. observed elevated levels of von Willebrand factor, t-PA and PAI-1, suggesting that these hemostatic alterations were a result of damage of endothelial cells[18]. Since our patients had no secondary complications, the increase in postheparin TFPI activity may be a reflection of early endothelial damage. There was a

significant difference between our diabetic subjects and controls with respect to von Willebrand factor and t-PA. This is in contrast with earlier reports, most of which suggested that von Willebrand factor and t-PA levels are only changed in diabetic patients with microvascular complications[16,18]. Because the levels of t-PA and von Willebrand factor in our patients were still within normal ranges, the exact significance of these altered levels is not clear. The differences in levels of von Willebrand factor could not be explained by a different distribution of blood group. There was no correlation between TFPI activity and von Willebrand factor. However, a significant correlation between t-PA and TFPI activity, when measured with the clotting assay, existed. The explanation for this finding is unclear, especially since the t-PA levels were significantly lower in the IDDM group in comparison with the controls. Although metabolic control may influence the levels of t-PA and von Willebrand[34,35], we found no relation between t-PA and von Willebrand factor and HbA_{1c}.

In conclusion, postheparin TFPI activity in IDDM patients without secondary complications is significantly increased, and correlates with the degree of metabolic derangement. The explanation for this observation is not yet known, but non-enzymatic glycation and AGE formation may play an important role. However, early damage of endothelial vascular cells as a direct result of hyperglycemia and oxidative stress can not be ruled out.

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Chapter 4

Tissue Factor Pathway Inhibitor (TFPI) release after heparin stimulation is increased in type 1 diabetic patients with albuminuria

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Abstract

Tissue factor pathway inhibitor (TFPI) is produced by and mainly bound to the vascular endothelium, probably by glycosaminoglycans. Heparin stimulates TFPI release from the endothelium. In uncomplicated patients with type 1 diabetes mellitus, we recently demonstrated higher basal and postheparin TFPI activity. In the present study, we investigated whether an increased TFPI activity could be resulted from an altered binding between TFPI and endothelial glycosaminoglycans. Nineteen uncomplicated type 1 diabetic patients (group I) were compared with 18 patients with retinopathy (group II), and 9 patients with retinopathy and albuminuria (group III). Blood samples were taken before (basal) and 30 minutes after 5,000 IU of heparin i.v. (postheparin). TFPI activity was measured chromogenically. Von Willebrand factor, t-PA, PAI-1 and thrombomodulin were also measured. Basal TFPI activity was higher in group III ($121\pm10\%$) compared to group II ($111\pm8\%$) or group I ($110\pm13\%$) ($p<0.05$), and strongly correlated with albuminuria ($r=0.66$, $p<0.05$). At all time points after heparin administration, TFPI activity in group III was significantly higher than in group I. TFPI activity was also higher in group III than in group II 5 to 30 minutes postheparin. The increase in postheparin TFPI activity, as measured as the incremental area under the curve, was higher in group III as compared to group I (65 ± 7 vs 59 ± 4; $p<0.05$). Of the other parameters, only thrombomodulin was higher in group III (44 ± 24 vs 26 ± 7 (group II) and 28 ± 9 ng/ml (group I); $p<0.01$). We conclude that basal and postheparin TFPI activity is increased in albuminuric patients. This suggests that the increase in TFPI activity could be the result of altered endothelial glycosaminoglycan characteristics.

Introduction

One of the main therapeutic goals in diabetes is to prevent or delay the development of macro- and microangiopathy. We therefore need instruments which enable us to estimate the risk of developing vascular complications early in the course of diabetes, so that specific preventive and therapeutic measures can be taken. Since damage of the endothelium precedes angiopathy, several investigators have looked for specific markers of endothelial dysfunction. Only when microvascular complications have already been established, von Willebrand factor (vWF), thrombomodulin, plasminogen activator inhibitor 1 (PAI-1) and tissue plasminogen activator (t-PA) have been identified as such markers[1-3]. The measurement of urinary albumin excretion is used as a substitute.

Tissue factor pathway inhibitor (TFPI), is produced by and mainly bound to the vascular endothelium[4]. TFPI is a coagulation inhibitor that inhibits factor Xa, and in complex with factor Xa, also the tissue factor/factor VIIa complex. The binding of TFPI to the endothelial cell surface is thought to be established by glycosaminoglycans like heparan sulfate[5,6]. The release of TFPI from the endothelium can be stimulated by heparin, a glycosaminoglycan for which TFPI has a much higher binding affinity than for heparan sulfate, which represents the majority of glycosaminoglycans at the endothelial cell surface[5,6]. We have previously demonstrated higher TFPI activity before and after heparin administration in type 1 diabetic subjects without vascular complications compared to healthy controls[7]. TFPI activity was strongly related to the degree of long-term metabolic derangement. Acute hyperglycemia did not affect TFPI activity in nondiabetic subjects[7]. Studies with cultured endothelial cells also indicate that chronic hyperglycemia increases release of TFPI after heparin stimulation[8]. In diabetic subjects with complications, we and others have demonstrated that basal TFPI activity is also increased[9-12]. In addition, it has been shown that basal plasma TFPI activity is increased in patients with manifest macroangiopathy, such as acute coronary disease[13,14]. TFPI activity is also altered in other pathological conditions, which are known to be associated with (micro)vascular abnormalities[15]. We recently showed that the increase in TFPI activity in type 1 diabetic patients is not the result of a compensatory response to a procoagulant state[11]. Alternatively, because of the binding to glycosaminoglycans, the increase in TFPI activity could be a result of altered functional properties of endothelial glycosaminoglycans, leading to a weaker binding of TFPI to glycosaminoglycans.

Subsequently to our earlier studies, we therefore investigated the functional relationship between TFPI and glycosaminoglycans by means of stimulation of TFPI release from the endothelium by heparin in type 1 diabetic patients with and without albuminuria.

Subjects, materials and methods

Forty-six type 1 diabetic patients were studied and an evaluation with respect to micro- and macrovascular complications (history, physical examination, urinary albumin excretion, ECG), diabetes control and lipid profile was performed in each subject. Patients with manifest cardiovascular disease (history of myocardial infarction, angina pectoris, positive ECG (Minnesota codes 1-1, 1-2, 8-1, 8-3), cerebrovascular accident and/or peripheral vascular disease) were excluded. Retinopathy was diagnosed by the ophthalmologist by means of fundoscopic examination. Microalbuminuria was defined as albumin excretion rate ≥ 3 mg/mmol creatinine in an early morning urine specimen on at least two consecutive occasions[16]. No patients use drugs that could influence hemostasis.
Twenty-seven type 1 diabetic patients with vascular complications (18 with retinopathy, 9 with retinopathy and albuminuria) were included in the present study. Nineteen type 1 diabetic patients free of complications, matched for age and metabolic control, served as controls. The characteristics of the subjects studied are depicted in Table 4.1. Part of these data has already been reported earlier[11].

Table 4.1 Demographic parameters of type 1 diabetic patients at baseline.

	DM without complications	DM with complications	
		Retinopathy	Retinopathy + albuminuria
	I	II	III
n	19	18	9
Sex ratio	11M/8F	9M/9F	3M/6F
Age (years)	39±8	42±9	39±10
Duration diabetes (years)	11 (2-32)*	22 (11-40)	23 (14-36)
Dosage insulin (IU/day)	48±16	45±12	49±11
Body mass index (kg/m^2)	23.8±4.5	25.4±2.6	22.6±1.7
$P_{Systolic}$ (mm Hg)	126±11	131±12	130±10
$P_{Diastolic}$ (mm Hg)	81±6	79±8	80±6

Data shown as mean ±SD, or median (range). * $p<0.001$ vs II and III.

Each study started at 08.00 a.m. while the subject was still fasting. In order to investigate the binding characteristics of TFPI to glycosaminoglycans at the endothelial cell surface, TFPI activity was measured before and after heparin administration. With the subject in supine position blood samples were taken via an indwelling catheter in a vein of the forearm just before and 3, 5, 10 and 30 minutes after an intravenous bolus injection of 5,000 IU of unfractionated heparin (Leo,

Denmark). For determining TFPI activity, vWF, thrombomodulin, t-PA and PAI-1, blood samples were collected in tubes containing sodium citrate 3.25% (dilution 1:10). Fasting blood samples were also drawn for determination of glycated hemoglobin (HbA_{1c}) and lipid profile at baseline. The protocol was approved by the Ethical Committee of the University Hospital, while written informed consent was obtained from all participants.

TFPI activity was measured using the chromogenic substrate assay according to Sandset after polybrene pretreatment (expressed in % with regard to standardized TFPI activity, measured in a plasma pool obtained from 45 healthy donors; CV 7.4% at 100% level in our laboratory)[17]. The increase in TFPI activity from baseline after heparin administration was also estimated by integral calculus as the incremental area under the curve (AUC). The results of the calculations of AUC are shown dimensionless. Plasma levels of vWF were determined by an ELISA method using rabbit anti-human vWF (Dako A/S, Denmark; CV 7%; normal range 60-180%). Thrombomodulin (Diagnostica Stago, France; CV 8%; mean 24 ng ml^{-1}) and t-PA antigen (Innogenetics, Belgium; CV 7%; normal range 1.3-10.4 ng ml^{-1}, mean 4.1±2.4 ng ml^{-1}) were measured by enzyme immunoassay. PAI-1 activity was photometrically measured (Kabi Diagnostica, Sweden; intra-assay CV 6%; normal range 1-20 AU ml^{-1}, median 8 AU ml^{-1}). HbA_{1c} was measured by column chromatography (Diamat, Bio-Rad, USA). Serum total and HDL-cholesterol and triglycerides were determined with enzymatic methods (Unimate 5 and 7, Roche, Basle, Switzerland), and apo A_1 and apo B were measured with immunoturbidimetry (Uni-kit, Roche). LDL-cholesterol was calculated with the Friedewald formula. Lipoprotein (a) was determined by a solid-phase, two-site immunoradiometric assay using two monoclonal antibodies directed toward different epitopes of apo(a) (Pharmacia Diagnostics, Uppsala, Sweden). At a Lp(a) concentration of 200 U l^{-1} the within-assay coefficient of variation is 4%. An enzymatic hexokinase method (Unimate 5, Roche) was used for measuring serum glucose concentrations. Serum free insulin was measured with a double antibody radioimmunoassay after polyethylene glycol pretreatment (Pharmacia Diagnostics; within-assay CV 3.4-6.1% in the range of 3-50 mU l^{-1}). For determination of urinary albumin an immunoturbidimetric technique was applied (Uni-kit, Roche; interassay CV 3%).

Statistics

All data are expressed as mean±SD or as median and range when not normally distributed. Because of repeated measurements in time a multiple analysis of variance (MANOVA) for repeated measurements was performed. For comparing more than two groups, one-way analysis of variance (ANOVA) with Student-

Newman-Keuls correction for multiple comparisons was used. The Kruskal-Wallis analysis of variance was applied if data were not normally distributed. Categorical variables were analyzed by the chi-square test. P-values ≤ 0.05 were considered statistically significant. The partial correlation coefficient was determined by stepwise multiple regression analysis.

Results

The mean duration of disease in the diabetic subjects without complications was shorter than in the patients with only retinopathy or with retinopathy and albuminuria (Table 4.1). However, the three groups did not differ with regard to gender, age, body mass index, blood pressure or daily insulin dosage. Three subjects with retinopathy and three with albuminuria received antihypertensive medication. There were no differences in smoking habits or use of alcohol. Regarding the metabolic parameters, the three groups were well-matched for HbA_{1c} (Table 4.2). Serum lipids, apo A_1 and B, lipoprotein (a) and insulin levels were similar in the three groups.

Table 4.2 Laboratory parameters of type 1 diabetic patients at baseline.

	DM without complications	DM with complications	
		Retinopathy	Retinopathy + albuminuria
	I	II	III
Glycated haemoglobin (%)	8.8±1.0	8.7±1.1	9.4±1.7
Total cholesterol (mmol/l)	5.0±0.7	5.6±0.8	5.5±1.0
Triglycerides (mmol/l)	1.10 (0.6-5.22)	1.01 (0.5-2.26)	1.33 (0.84-2.42)
HDL-cholesterol (mmol/l)	1.35±0.43	1.51±0.43	1.34±0.2
LDL-cholesterol (mmol/l)	3.01±0.79	3.59±0.72	3.51±1.1
Apo A_1 (g/l)	1.60±0.28	1.65±0.26	1.65±0.22
Apo B (g/l)	1.01±0.21	1.10±0.19	1.19±0.25
Lipoprotein (a) (U/l)	105 (9-857)	147 (25-622)	109 (34-907)
Serum insulin (mU/l)	7.0 (3.0-42.4)	8.1 (4.5-12.4)	8.2 (6.9-43.9)
AER (mg/mmol creatinine)	0.5 (0-2.7)	0.4 (0-1.6)	20 (5.6-38.0)*

Data shown as mean ±SD, or median (range). AER: albumin excretion rate. * $p < 0.0001$ vs I and II.

The endothelium-dependent hemostatic parameters vWF and t-PA were not different in the three groups (Table 4.3).

Table 4.3 Hemostatic parameters of type 1 diabetic subjects at baseline.

	DM without complications	DM with complications	
		Retinopathy	Retinopathy + albuminuria
	I	II	III
Von Willebrand factor (%)	132±42	118±41	130±40
Thrombomodulin (ng/ml)	28±9	26±7	44±24*
t-PA (ng/ml)	2.81±1.26	3.01±1.44	2.77±1.34
PAI-1 (AU/ml)	3 (0-15)	2 (0-6)	1 (0-8)§

Data shown as mean ±SD, or median (range). * $p<0.05$ vs I and II; § $p<0.05$ vs I

Thrombomodulin levels were significantly higher ($p<0.05$) in the albuminuric patients, compared to the patients with only retinopathy or without complications. PAI-1 activity was lower in the patients with albuminuria in comparison with the uncomplicated patients ($p<0.05$). However, the PAI-1 levels were within the normal range in all three groups. Basal TFPI activity was significantly higher in the patients with albuminuria, compared to the patients with only retinopathy or those without complications (121±10% vs 111±8% and 110±13% respectively, $p<0.05$; Figure 4.1). A positive correlation between basal TFPI and albuminuria was found ($r=0.66$, $p<0.05$; Figure 4.2), while no correlation between basal TFPI and HbA_{1c} existed. TFPI activity in the albuminuric group was significantly higher at all time points after the bolus injection of heparin, in comparison with the uncomplicated subjects ($p<0.01$). Compared to the patients with retinopathy, the albuminuric patients had higher TFPI activity from 5 to 30 minutes postheparin ($p<0.05$; Figure 4.1). The increase in postheparin TFPI activity, as measured as the incremental area under the curve (AUC), was higher in the albuminuric group than in the uncomplicated group (65±7 vs 59±4, $p<0.05$). This increase in postheparin TFPI activity was independent of the TFPI activity at baseline. In contrast to basal TFPI activity, a weak correlation ($r=0.39$, $p<0.01$) between postheparin TFPI activity and HbA_{1c} could be demonstrated. No correlation between the other endothelium-dependent parameters and basal TFPI activity was found, while postheparin TFPI activity was negatively correlated with vWF ($r=-0.38$, $p<0.01$). Stepwise multiple regression analysis revealed albuminuria to be the only independent parameter associated with basal TFPI activity in the complicated patients (partial correlation coefficient: 0.56, $p<0.001$).

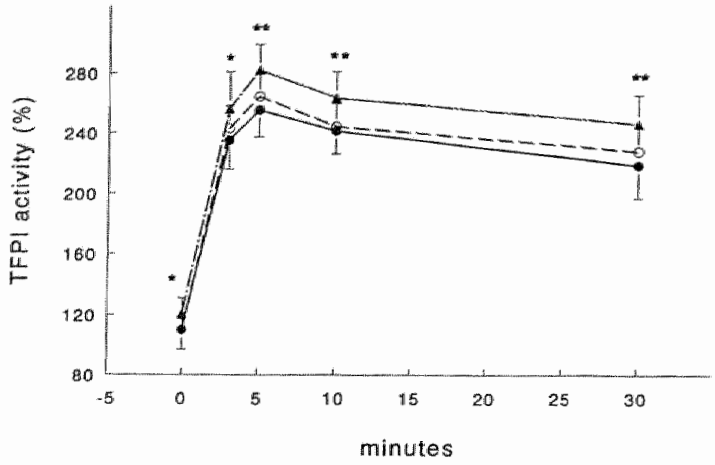

Figure 4.1 TFPI activity, before and after an i.v. bolus injection of 5,000 IU of heparin in uncomplicated type 1 diabetic patients (●; n=19), patients with only retinopathy (○; n=18) or with retinopathy and albuminuria (▲; n=9). Basal TFPI activity is significantly higher in the albuminuric group, compared to the uncomplicated and retinopathic group (* $p<0.05$). TFPI activity is also significantly higher in the patients with albuminuria at all time points after heparin administration (* $p<0.05$; ** $p<0.01$ vs uncomplicated, and $p<0.05$ vs retinopathic group). The increase in TFPI activity is independent of basal TFPI activity.

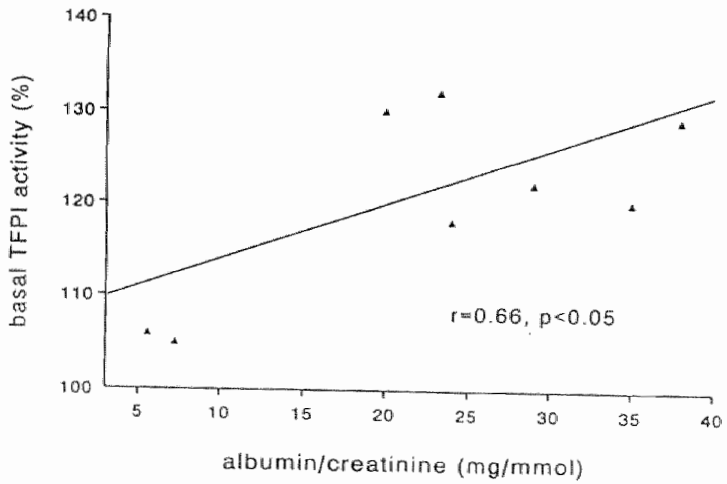

Figure 4.2 Correlation between basal TFPI activity and albuminuria.

Discussion

In the present study, we demonstrated significantly higher basal and postheparin TFPI activity in type 1 diabetic patients with retinopathy and albuminuria, compared to patients with only retinopathy or without complications. The increase in TFPI activity after heparin administration was independent of basal TFPI activity. The major determinant of these increased levels of TFPI was albuminuria. Simultaneously, thrombomodulin levels were significantly increased in the albuminuric patients, while no differences in the levels of von Willebrand and t-PA were found.

In an earlier study, we demonstrated that an increased basal TFPI activity in type 1 diabetic patients does not result from a reaction to a prethrombotic state[11]. Because TFPI is produced by and mainly bound to the vascular endothelium, it may reflect endothelial function. At the endothelial cell surface, TFPI is probably bound to glycosaminoglycans, such as heparan sulfate[5,6]. In comparison with retinopathy, (micro)albuminuria is more consistently associated with generalized angiopathy in diabetic subjects[18,19]. It is suggested that alterations in endothelial intracellular enzymes, involved in the metabolism of glycosaminoglycans, constitute the primary cause of albuminuria and the associated complications[18]. Diabetes affects heparan sulfate metabolism[20,21], partly by the inhibition of glucosaminyl N-deacetylase, a key enzyme in heparan sulfate synthesis, due to hyperglycemia[22]. Genetic defects in the regulation of glycosaminoglycan production, resulting in a decreased synthesis, have also been suggested to exist in diabetic patients with nephropathy[23]. The loss of heparan sulfate, the main glycosaminoglycan component of basement membranes of glomeruli, will lead to loss of anionic sites, and subsequently albuminuria may develop[24,25]. Indeed, it has been shown in type 1 diabetic patients with nephropathy that heparan sulfate is decreased within the glomerular basement membrane[26]. In animals with streptozotocin-induced diabetes, a reduction in the synthesis of heparan sulfate by endothelial cells has also been demonstrated[27]. A decrease in the number of endothelial glycosaminoglycan binding sites for TFPI could result in higher levels of circulating TFPI, explaining the increased basal TFPI activity in the plasma of our albuminuric diabetic subjects. Increased unstimulated TFPI activity has also been found in patients with diabetic nephropathy by others[9,10].

Not only synthesis, but also function and/or structure of endothelial glycosaminoglycans could be altered in diabetes. Glycosaminoglycans are negatively charged due to the sulfate groups present. Inhibition of glucosaminyl N-deacetylase also results in the synthesis of low-sulfated heparan sulfate molecules[28], and a reduced negative surface charge on the arterial endothelium in diabetic rats has been demonstrated[27]. This may explain the increased transcapillary leakage of albumin in type 1 diabetic patients with microalbuminuria[29]. TFPI is bound to glycosaminoglycans with a highly positively charged COOH-terminus, a

binding of electrostatic nature[6]. The increased postheparin TFPI activity in our albuminuric patients may therefore be explained by a weaker binding. As we have suggested earlier, alterations in synthesis and function of glycosaminoglycans could be the result of processes like nonenzymatic glycation, formation of advanced glycated endproducts and oxidative stress[7].

It is reported that concentrations of thrombomodulin, a membrane protein of the vascular endothelial cell, is increased in diabetic patients with albuminuria, suggesting generalized angiopathy to be present in this patient group[2,30]. We confirmed this finding in our patients. Levels of PAI-1 were lower in the albuminuric group as compared to the uncomplicated group. However, because all the PAI-1 levels were within normal range in the three subgroups, the meaning of this finding is not clear. On the other hand, levels of t-PA and vWF were not different between our three patient groups. In the literature, some authors reported increased levels of PAI-1, vWF and t-PA, while others have observed normal or even decreased levels in diabetic patients with or without (micro)albuminuria[1,31,32]. An explanation for these different observations may be a difference in metabolic control reported in the various studies. It is known that the levels of t-PA, PAI-1 and vWF are influenced by glucose metabolism[33-35]. Several studies indicate poorer metabolic control in subjects with nephropathy[1,35]. In contrast, our patients were carefully matched for glycemic control. In addition, it is also known that these endothelium-dependent parameters are affected by the presence of hypertension[36-38]. Blood pressure was similar in our patients with or without complications. A third explanation may be the use of antihypertensive medication. Such therapy has a favourable effect on fibrinolytic factors and vWF[36,38]. Three of our patients with retinopathy and three with albuminuria used antihypertensive drugs. When these subjects were excluded from statistical analysis, there were still no significant changes in the levels t-PA, PAI-1 or vWF in the three groups. One prospective study showed a rise in the levels of vWF, in close relation to the development and progression of albuminuria[39]. However, at the start of this study, there was a difference in blood pressure and metabolic control between the group that remained normoalbuminuric and the group that developed albuminuria. In the analysis and interpretation of the data in earlier studies, blood pressure and antihypertensive therapy have not frequently been taken into account as possible confounders. Hence, the former observations regarding these endothelium-dependent factors must be interpreted cautiously.

In our earlier study we have shown that TFPI is strongly correlated with long-term glycemic control as measured by HbA_{1c}[7]. In the present study, we therefore matched the patients for their degree of metabolic control, so that HbA_{1c} could not contribute as a confounding factor. It appeared that basal TFPI activity did not correlate with HbA_{1c}, while there was only a weak correlation between postheparin TFPI activity and metabolic control. In addition, levels of serum lipids did not

differ between the three patients groups, and in contrast to observations by others[40,41], we were unable to find correlations between TFPI activity and serum lipids and apolipoproteins.

In summary, type 1 diabetic patients with albuminuria show increased basal and postheparin TFPI activity. Because TFPI is an endothelium-dependent glycoprotein, bound to glycosaminoglycans, these findings suggest that TFPI activity could reflect disturbances in endothelial glycosaminoglycans. TFPI may therefore be considered an marker of endothelial (functional) alterations. The finding of increased thrombomodulin levels, a strong positive correlation between albuminuria and basal TFPI activity, and an increase in postheparin TFPI activity in this patient group, support this hypothesis. We postulate that alterations in metabolism and function of glycosaminoglycans, as seen in diabetes, are responsible for the increased TFPI activity in albuminuric patients. Whether TFPI activity could be an important factor with respect to diagnosing early vascular abnormalities, has still to be proven in future prospective studies.

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Chapter 5

Increased Tissue Factor Pathway Inhibitor (TFPI) and coagulation in patients with insulin-dependent diabetes mellitus

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Abstract

Recently, we found an increase in tissue factor pathway inhibitor (TFPI) activity in patients with insulin-dependent diabetes mellitus (IDDM). This increase in TFPI activity could be the result of increased thrombin formation and/or altered binding of TFPI to glycosaminoglycans. We studied TFPI activity (chromogenic assay) in relation to prothrombin F_{1+2} fragments and endogenous thrombin potential (ETP), in 46 IDDM patients, and 18 age and sex-matched healthy controls. Prothrombin, antithrombin and thrombomodulin were also determined. In IDDM patients, TFPI activity and F_{1+2} levels were significantly higher, while ETP, prothrombin antigen levels, and antithrombin activity were lower as compared to the controls. In IDDM patients with microalbuminuria, a manifestation of generalized angiopathy, TFPI activity, F_{1+2} and thrombomodulin levels were higher than in patients with only retinopathy or patients without complications. No correlation between TFPI activity, F_{1+2} levels and thrombomodulin was found, while TFPI activity was negatively correlated with ETP (r-0.27). Microalbuminuria was significantly correlated with TFPI activity ($r=0.46$), F_{1+2} ($r=0.56$), and thrombomodulin ($r=0.52$). In TFPI-depleted plasma, ETP increased, indicating that ETP is affected by TFPI. In conclusion, the increase in TFPI activity in IDDM patients may not be considered to be a reaction on a procoagulant state. It is hypothesized that vascular damage, leading to alterations in glycosaminoglycans, is in part responsible for the changes in TFPI activity, F_{1+2} levels and ETP.

Introduction

It is well known that the vascular endothelium plays an essential role in the regulation of (local) hemostatic processes[1]. The major defence mechanisms that prevent inappropriate coagulation are found at the endothelial cell surface[2]. The endothelium itself is known to produce procoagulant factors like von Willebrand factor, and anticoagulant factors, such as heparan sulfate and thrombomodulin[3,4]. In diabetic patients a procoagulant state is observed, which could contribute to the risk of premature cardiovascular events. This hypercoagulable state could be caused by a dysbalance between hemostatic factors in plasma, and/or at the endothelial cell surface. So far, indirect evidence for a state, favouring thrombin formation, has been gathered, mainly using plasma parameters like fibrinogen and antithrombin activity, or endothelium-dependent parameters like von Willebrand factor[3,4]. More recently, it is suggested that increased levels of factor VIIa may produce hypercoagulability in diabetic patients with endothelial dysfunction[5]. Impaired fibrinolysis could also contribute to a dysbalance in hemostasis in diabetes[4]. Studies with human endothelial cells cultured under hyperglycemic conditions have shown prethrombotic alterations at the endothelial cell surface[6]. In recent years, prothrombin fragments F_{1+2}, which are the result of thrombin formation, are used for determining thrombin generation in vivo[7]. It is now also possible to assess thrombin generation in plasma ex vivo after activation of plasma by tissue factor, using the sensitive endogenous thrombin potential (ETP) according to Hemker[8].
A new Kunitz-type coagulation inhibitor, tissue factor pathway inhibitor (TFPI), has been recently identified[9]. It is produced by and mainly bound to the vascular endothelial cells, probably by glycosaminoglycans[10-12]. Only about 5% of the TFPI in the plasma circulates as a free, uncomplexed protein. TFPI inhibits factor Xa and in complex with factor Xa also the tissue factor/factor VIIa complex. TFPI activity is altered in several pathological conditions[13-15]. Its release from the endothelium can be stimulated by glycosaminoglycans like heparin[16]. Although disturbances in hemostatic parameters in diabetic patients are involved in the development of cardiovascular complications[17-20], little is known about TFPI activity in diabetic subjects. In non-insulin-dependent diabetic (NIDDM) patients with microalbuminuria increased levels of TFPI have been found[5]. We recently demonstrated that basal and postheparin TFPI activity was significantly higher in uncomplicated insulin-dependent diabetic (IDDM) subjects than in healthy controls[21]. A strong correlation with metabolic control was found. The explanation for these findings is not known, but alterations in binding characteristics of TFPI to glycosaminoglycans, due to glycosylation or oxidative stress could play a role. However, the increase in TFPI activity could also be secondary to increased (local) formation of thrombin. In the present study, we therefore examined basal TFPI

activity in IDDM patients, with and without complications, in relation to the degree of coagulation, measured by F_{1+2} fragments, antithrombin activity and thrombomodulin. In addition, we studied the ETP in relation to TFPI activity.

Materials and methods

IDDM patients were evaluated with respect to micro- and macrovascular complications (history, physical examination, fundoscopy by ophthalmologist, urinary albumin excretion, ECG) and diabetes control. Retinopathy was diagnosed by the ophthalmologist by means of fundoscopic examination. Microalbuminuria was defined as albumin excretion rate ≥ 3 mg/mmol creatinine in an early morning urine specimen on at least two consecutive occasions[22]. Patients with manifest cardiovascular disease (history of myocardial infarction, angina pectoris, positive ECG (Minnesota codes 1-1, 1-2, 8-1, 8-3), cerebrovascular accident and/or peripheral vascular disease) were excluded. No patients use drugs that could influence hemostasis. In addition, 18 age- and sex-matched healthy subjects recruited from a group of blood donors, served as controls.
Twenty-seven IDDM patients with vascular complications (18 with retinopathy, 9 with retinopathy and micro-/macroalbuminuria) were studied. Nineteen IDDM patients without microangiopathy were matched for age and metabolic control. The same patient population was used as described in the study of chapter 4. The characteristics of the subjects studied are depicted in Table 5.1.

Table 5.1 Demographic and hemostatic characteristics of healthy controls and IDDM patients.

	Controls	IDDM
n	18	46
Sex ratio (males/females)	11M/7F	23M/23F
Age (years)	36±5	40±9
TFPI (%)	99±18	112±11*
$F_1 + F_2$ (nmol/l)	0.63±0.17	0.94±0.43**
ETP (nM.min)	365±52#	339±57
Prothrombin (%)	116±12**	97±13
Antithrombin (%)	99±7*	91±11

Data shown as mean ±SD. * $p<0.01$; ** $p<0.001$; # $p<0.05$

For determining TFPI activity, prothrombin fragments F_{1+2}, and the ETP blood samples were collected in tubes containing sodium citrate 3.25% (dilution 1:10).

Blood samples were also drawn for determination of glycated hemoglobin in the diabetic subjects. Glycated hemoglobin (HbA_{1c} fraction) was measured by column chromatography (Diamat, Bio-Rad, USA), the remainder of the blood samples were centrifuged and plasma was stored at -70°C until further processing. The protocol was approved by the Ethical Committee of the University Hospital, while written informed consent was obtained from the participants.

TFPI activity was measured using the chromogenic substrate assay according to Sandset (expressed in % with regard to standardized TFPI activity, measured in a plasma pool obtained from 65 healthy donors; CV 7.4% at 100% level)[23]. In vivo thrombin generation was assessed by determination of prothrombin fragments F_{1+2} using ELISA (Enzygnost Behring, Marburg, Germany; intra-assay CV 5-7.5%, interassay CV 6-13%). Ex vivo thrombin formation, following activation of the tissue factor pathway of coagulation, was measured using the computer assisted technique according to Hemker (endogenous thrombin potential (ETP); CV 2.6%)[8]. In short, defibrinated plasma was obtained by mixing an aliquot of plasma with 1:50 volume of a reptilase solution, letting a clot form for 10 minutes at 37°C and keeping the clotted plasma for 10 minutes at 0°C. The fibrin formed was discarded by winding it on a small plastic spatula. The concentrations of the coagulation factors did not change significantly by the reptilase treatment[24]. The ETP was measured semi-automatically (Cobas Fara, Hoffmann-La Roche). To 80 μl of defibinated plasma 20 μl of recombinant tissue factor (Dade, USA) diluted 1:1 in Hepes buffer was added. After an incubation period of at least 30 seconds at 37°C, thrombin generation was started by the addition of 20 μl of a solution of chromogenic substrate SQ 68 (3 mM) and calcium (0.1 M). The substrate conversion was monitored by a continuous measurement of the optical density at 405 nm during 15 minutes. From the optical density-time curves the ETP was calculated in nM.min by computer. The area under the thrombin generation curve represents the amount of substrate that thrombin, generated in the plasma, can potentially convert. Prothrombin antigen levels were measured nephelometrically, using polyclonal rabbit antihuman coagulation factor II (CLB, Amsterdam, The Netherlands; CV 5%). Antithrombin activity was determined by a chromogenic assay (Coamatic antithrombin, Chromogenix, Sweden; CV 4%).

In order to assess the influence of TFPI on the thrombin generation ex vivo, we depleted normal plasma of a healthy volunteer from TFPI using polyclonal anti-TFPI IgG coupled to Sepharose (1.5 mg IgG/ml; kindly provided by Nordfang, Novo Nordisk, Danmark). The antibody has been affinity purified on $TFPI_{1-161}$, whereas the titer is approximately 3000 "Bethesda like" U/mg. Before and after depletion, the thrombin generation in 4 subsamples was measured. In another experiment, we added polyclonal goat-antihuman TFPI IgG antibodies (3000 U/ml; courtly received from Nordfang) to a sample of the normal plasma pool, and measured the thrombin generation in 4 subsamples before and after adding TFPI

antibodies. TFPI activity after both TFPI depletion and treatment with TFPI antibodies was chromogenically measured. Urinary albumin was measured by an immunoturbidimetric technique (Uni-kit, Roche; interassay CV 3%).

Statistics

All data are expressed as mean ±SD or as median and range when not normally distributed. For comparing more than two groups, one-way analysis of variance (ANOVA) with Student-Newman-Keuls correction for multiple comparisons was used. The Kruskal-Wallis analysis of variance was applied if data were not normally distributed. Categorical variables were analyzed by the chi-square test. P-values ≤ 0.05 were considered statistically significant. The partial correlation coefficient was determined by stepwise multiple regression analysis.

Results

There was no difference in age or gender between the healthy control and diabetic group or subgroups (Table 5.1 and 5.2).

Table 5.2 Demographic and hemostatic parameters of IDDM patients with and without complications.

	No complications	Retinopathy	Retinopathy + albuminuria
	I	II	III
n	19	18	9
Sex ratio (males/females)	11M/8F	9M/9F	3M/6F
Age (years)	39±8	42±9	39±10
Glycated hemoglobin (%)	8.8±1.0	8.7±1.1	9.4±1.7
TFPI (%)	110±13	111±7	121±10*
F_{1+2} (nmol/l)	0.74±0.21	0.82±0.19	1.60±0.50**
ETP (nM.min)	357±72	318±30	348±56
Prothrombin (%)	98±13	92±12	105±15
Antithrombin (%)	90±10	90±12	94±11
Thrombomodulin (ng/ml)	28±9	26±7	44±24*

Data shown as mean ±SD. * $p<0.05$ vs I and II; ** $p<0.0001$ vs I and II.

The three diabetic subgroups did not differ in HbA_{1c}. Basal TFPI activity was significantly higher in the diabetic subjects as compared to the healthy controls ($112\pm11\%$ vs $99\pm18\%$, $p<0.01$). Compared to the patients without complications and with only retinopathy, TFPI activity was higher in the albuminuric patients (Figure 5.1).

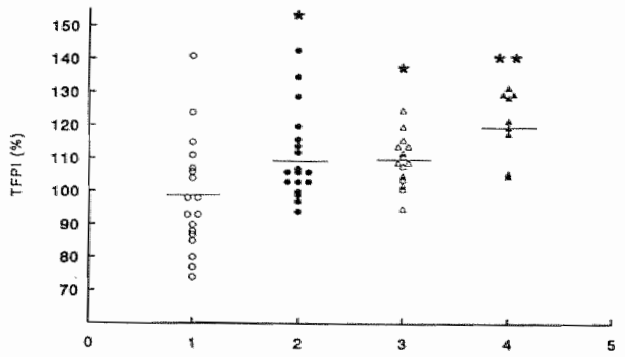

Figure 5.1 TFPI activity was significantly increased in the diabetic subgroups in comparison with the healthy controls (○). In the patients with microalbuminuria (▲), TFPI activity was significantly higher than in the patients with only retinopathy (Δ) or without complications (●). * $p<0.05$ vs healthy controls , ** $p<0.01$ vs healthy controls, $p<0.05$ vs patients with only retinopathy or without complications.

For the prothrombin fragments F_{1+2}, equal results were seen. F_{1+2} levels were significantly higher in the diabetic group than in the control group (0.94 ± 0.43 nmol/l vs 0.63 ± 0.17 nmol/l, $p<0.001$), and were higher in the patients with albuminuria, compared to the patients with only retinopathy and uncomplicated patients (Table 5.2). The ETP was significantly higher in the controls than in the diabetic subjects (373 ± 50 nM.min vs 339 ± 57 nM.min, $p<0.01$). However, in the three diabetic subgroups, the ETP was similar. Levels of prothrombin antigens were lower in the diabetic group than in the healthy control group ($97\pm13\%$ vs $116\pm12\%$, $p<0.001$), while there were no differences between the diabetic subgroups. Antithrombin activity was also decreased in the diabetic patients in comparison with the healthy subjects ($91\pm11\%$ vs $99\pm7\%$, $p<0.01$). The antithrombin activity was not different between the diabetic subgroups. Thrombomodulin was higher in the microalbuminuric group (44 ± 24 g/ml, $p<0.05$). TFPI activity negatively correlated with the thrombin potential ($r=-0.27$, $p<0.05$)), while no correlation with F_{1+2} or thrombomodulin was found. There was also no correlation between F_{1+2} and the thrombin potential. However, prothrombin was positively correlated with the ETP ($r=0.77$, $p<0.001$) and antithrombin activity ($r=0.25$, $p<0.05$). In the diabetic patients, micro-

albuminuria was positively correlated with TFPI activity ($r=0.46$, $p<0.01$), F_{1+2} levels ($r=0.56$, $p<0.001$), and thrombomodulin ($r=0.52$, $p<0.001$). When performing stepwise multiple regression analysis it appeared that only microalbuminuria (partial $r=0.56$, $p<0.001$) and the thrombin potential (partial $r=-0.06$, $p<0.05$) were associated with TFPI activity, while microalbuminuria was the only determinant of the F_{1+2} levels (partial $r=0.51$, $p<0.001$). When normal plasma was depleted from TFPI, an increase in ETP was seen (from 443 ± 2 to 490 ± 4 nM.min; Figure 5.2). An increase was also found when normal plasma was treated with TFPI-antibodies (from 390 ± 4 to 447 ± 1 nM.min). When measured chromogenically, TFPI activity after TFPI depletion and treatment with TFPI antibodies was less than 1%.

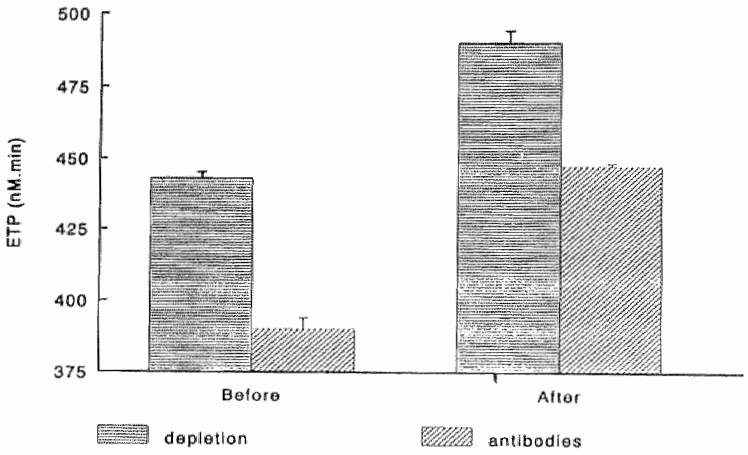

Figure 5.2 Influence of TFPI activity on the ETP in two separate experiments (see Materials and methods). An increase in ETP is seen after TFPI depletion or treatment with TFPI antibodies of normal plasma.

Discussion

In the present study, we examined the possible relationship between coagulation and TFPI activity in healthy and diabetic subjects. A procoagulant state was assessed by measuring the prothrombin fragments F_{1+2} (in vivo) and the ETP (ex vivo). It appeared that TFPI activity and F_{1+2} levels were increased in diabetic patients as compared to healthy controls. On the other hand, the ETP was decreased. However, no correlation between TFPI activity and F_{1+2} levels was found, while a negative correlation between TFPI activity and the ETP existed. In

the microalbuminuric patients, TFPI activity, F_{1+2} levels, and thrombomodulin were higher than in the patients with only retinopathy or without complications. Microalbuminuria was strongly correlated with TFPI activity, F_{1+2} levels, and thrombomodulin.

In an earlier study, we demonstrated an increased TFPI activity in diabetic patients[21]. From the data of the present study, it can be concluded that this seems especially to be true for diabetic subjects with microalbuminuria. In contrast to retinopathy, microalbuminuria is more consistently associated with generalized angiopathy[25,26]. It is suggested that alterations in enzymes, involved in the metabolism of glycosaminoglycans constitute the primary cause of albuminuria and the associated complications[25]. Because TFPI is mainly bound to the vascular endothelium, probably by glycosaminoglycans, this could suggest that increased TFPI activity reflects endothelial dysfunction. Because TFPI is a coagulation inhibitor, this increase could also be a compensatory reaction on a procoagulant state, as can be found in diabetes. Presently, not only TFPI activity, but also F_{1+2} levels were increased in the diabetic subjects as compared to the healthy controls. F_{1+2} are formed during thrombin formation, while antithrombin, in complex with glycosaminoglycans, decreases F_{1+2} formation by inhibiting the generation of thrombin[27]. In diabetic subjects, one of the potential defects in the natural anticoagulant mechanisms that might lead to elevated F_{1+2} levels could be a decreased synthesis of glycosaminoglycans like heparan sulfate by endothelial cells[28]. A diminished synthesis of endothelial glycosaminoglycans is likely to reduce the activity of the endogenous heparan sulfate-antithrombin mechanism[27]. In addition, it has been shown that antithrombin activity is decreased by nonenzymatic glycation[29]. In agreement with these data, we found a decreased antithrombin activity in our diabetic patients in comparison with the healthy controls. We believe that the increase in TFPI activity in the present study does not result from a prethrombotic state, as indicated by increased levels of F_{1+2}. However, the increase of TFPI activity and F_{1+2} levels could be the result of vascular endothelial damage leading to alterations in glycosaminoglycans. This hypothesis is supported by the finding that TFPI activity, F_{1+2} levels and thrombomodulin are increased in the microalbuminuric patients as compared to the retinopathic and uncomplicated patients. In addition, TFPI activity, F_{1+2} and thrombomodulin were positively correlated with microalbuminuria, while no relation between TFPI activity and F_{1+2} or thrombomodulin was found.

Although an increase in F_{1+2} levels indicates an in vivo state, favouring clot formation, we could not confirm this by the ex vivo measurement of the thrombin potential in plasma, which was decreased in the diabetic patients. A negative correlation between TFPI activity and the ETP was found. The possible influence of TFPI on the generation of thrombin was therefore further explored by assessing

the ETP in TFPI depleted plasma and plasma treated with TFPI antibodies. A decrease in TFPI activity resulted in an increase in ETP. From these data, we conclude that the decreased ETP in diabetic patients is, at least in part, caused by an increased TFPI activity in plasma. The discrepancy between the F_{1+2} levels and the ETP underlines the importance of the endothelium in the pathogenesis of clotting disturbances leading to cardiovascular events in diabetes.

The thrombin generation is also affected by prothrombin levels and antithrombin activity. Prothrombin levels were lower in the diabetic group as compared to the controls, which could result in a decreased ETP. On the other hand, antithrombin activity was also decreased in our diabetic subjects, which could lead to an increased ETP. We found a decrease in ETP, suggesting a predominant effect of lower prothrombin levels and higher TFPI activity on the ETP in our diabetic subjects.

Thrombomodulin, a membrane proteoglycan of the vascular endothelial cell, provides binding sites for thrombin[30]. In complex with thrombomodulin, thrombin loses its procoagulant properties, and this will result in an accelerated inhibition of formation and inactivation of thrombin by antithrombin[27]. Diabetes is characterized by the formation of advanced glycation endproducts (AGEs). It has been shown that the endothelial cell surface thrombomodulin activity is decreased by AGEs[31]. In addition, thrombomodulin is considered to be a marker of endothelial damage, because of increased plasma levels of thrombomodulin in diabetic patients with nephropathy[18]. We could confirm this in our microalbuminuric patients, in whom the plasma thrombomodulin levels were increased compared to the other diabetic groups. A decrease in thrombomodulin activity and thrombin binding sites could also lead to increased F_{1+2} levels. This may also explain the finding that the microalbuminuric patients with increased TFPI activity showed no decreased ETP as compared to the other diabetic subgroups. The effect of the increased TFPI activity on ETP is probably neutralized by an decreased activity of endothelial cell surface thrombomodulin activity. The finding that antithrombin activity and prothrombin antigen levels did not differ between the diabetic subgroups supports this hypothesis.

In conclusion, the increase in TFPI activity in IDDM patients may not be considered a compensatory reaction on a procoagulant state as measured by an increase in F_{1+2}. Besides, the ETP is decreased in IDDM subjects as compared to non-diabetic controls, partly because of an increment of TFPI activity and lower prothrombin levels seen in diabetic patients. A procoagulant state could be the result of disturbances in hemostatic factors in plasma and/or at the endothelial cell surface. We postulate that an increase in TFPI activity in diabetes does not result from a prethrombotic state in plasma, but probably reflects vascular endothelial damage, resulting in alterations in glycosaminoglycans and/or binding properties of

TFPI to glycosaminoglycans. To explain the association between diabetes and the occurrence of premature cardiovascular events, it is hypothesized that an local decrease in anticoagulant surface activity of TFPI at the site of endothelial damage is important, especially in a procoagulant surrounding. In this respect, the value of the ETP has not yet been proven, and therefore needs further investigation.

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Chapter 6

Tissue factor pathway inhibitor and other endothelium-dependent hemostatic factors in the elderly with normal or impaired glucose tolerance and type 2 diabetes mellitus

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Abstract

Impaired glucose tolerance (IGT) is believed to be a prediabetic phase, which precedes the development of type 2 diabetes mellitus. In elderly subjects, IGT and diabetes are both independently associated with the occurrence of cardiovascular disease. Because endothelial damage precedes atherosclerotic changes of the vascular wall, several markers of endothelial dysfunction, eg von Willebrand factor (vWF), tissue plasminogen activator (t-PA), plasminogen activator inhibitor type-1 (PAI-1) and thrombomodulin, were studied in a population-based study concerning elderly subjects with normal (NGT) or impaired glucose tolerance and type 2 diabetes mellitus. Besides these endothelium-dependent factors, we also investigated tissue factor pathway inhibitor (TFPI) activity in relation to metabolic parameters and cardiovascular risk factors. In earlier reports, TFPI activity has been shown to be increased in diabetic subjects, especially with albuminuria. All data were adjusted for age. Increased levels of vWF antigen, t-PA antigen and PAI-1 activity were seen in the IGT and diabetic group as compared to the NGT group. TFPI activity and thrombomodulin levels were increased in all elderly subjects with no differences between the groups. There was a positive association between HbA_{1c} and TFPI activity and vWF antigen. Fasting blood glucose correlated with vWF antigen, t-PA antigen and PAI-1 activity, while urine albumin excretion correlated with TFPI activity, vWF antigen and PAI-1 activity. Serum insulin levels correlated strongly not only with vWF antigen and t-PA antigen, but with PAI-1 activity as well. This correlation did not change after further adjustment for serum glucose and HbA_{1c}, which may suggest that in the elderly subjects impaired fibrinolysis is probably associated with insulin resistance. There were no associations between the endothelium-dependent hemostatic factors and lipids, except for a negative correlation between HDL-cholesterol and thrombomodulin. It is concluded that in elderly subjects with IGT several endothelium-dependent hemostatic factors are already consistently increased, indicating endothelial damage in this stage.

Introduction

The state of impaired glucose tolerance (IGT) is considered to be a transitional phase to the development of type 2 diabetes mellitus. The prevalence of IGT and type 2 diabetes is increasing with age[1-3]. Not only ageing itself is a risk factor for atherosclerosis, but IGT and diabetes as well are both independently associated with the occurrence of cardiovascular disease[4,5]. There are several factors that contribute to the development of cardiovascular disease in diabetic and nondiabetic subjects, such as dyslipidemia, (micro)albuminuria and hypertension[6-12]. Hyperinsulinemia as an independent risk factor is controversial[13]. It has been shown that endothelial damage precedes atherosclerotic changes of the vascular wall. Therefore, several markers of endothelial (dys)function like von Willebrand factor (vWF), tissue plasminogen activator (t-PA), plasminogen activator inhibitor type-1 (PAI-1) and thrombomodulin, have been studied in the past, in order to assess whether they can predict the risk for developing cardiovascular disease[14,15]. Recently, we and others have demonstrated increased tissue factor pathway inhibitor (TFPI) activity in diabetic patients[16,17]. Particularly, this seems to be true in diabetic patients with (micro)albuminuria[17,18]. Because TFPI is mainly produced by and bound to the vascular endothelium, possibly by glycosaminoglycans[19], these data suggest that TFPI may also reflect endothelial (dys)function. Besides, TFPI is also partly associated with lipoproteins[20,21]. In a population-based study, we studied TFPI activity and other endothelium-dependent factors in elderly subjects with normal glucose tolerance (NGT), IGT, and diabetes type 2.

Subjects, material and methods

The study was conducted in participants of the Rotterdam Study, a population-based prospective cohort study of determinants of chronic disabling diseases in the elderly[22]. The baseline examinations were conducted between 1990 to 1993, and included 7983 subjects (response rate 78%). Informed consent was obtained from all subjects, and the study was approved by the medical ethics committee of the Erasmus University Medical School. The participants were evaluated with respect to micro- and macrovascular complications (history, physical examination, urinary albumin excretion, ECG), diabetes control and lipid profile. Grade of retinopathy was assessed by an ophthalmologist by means of fundoscopic examination. During the first follow-up examination of the Rotterdam Study in 1993-1994, a sample of participants was invited from those age 55 to 75 years at baseline for an additional diabetes examination, including an oral glucose tolerance test. The overall response rate for the follow-up examination was 90%; 1107 subjects participated in the diabetes study. The present study population consists of a random sample of 144

men and women, who were divided according to their glucose tolerance at follow-up, using the WHO criteria, into a group with normal glucose tolerance, impaired glucose tolerance and diabetes. The subjects did not use medication, that could influence hemostasis. The characteristics of the subjects studied are depicted in Table 6.1.

Table 6.1 Characteristics of subjects with normal and impaired glucose tolerance, and type 2 diabetes.

	Normal	IGT	Type 2 diabetes
Gender (male:female)	18M : 33F	23M : 25F	29M : 18F
Age (years)	65±6	69±6*	69±6*
Body mass index (kg/m^2)	25.5±3	28.0±3.5*	27.3±3.3*
Systolic blood pressure (mm Hg)	133±23	150±20*	146±21*
Diastolic blood pressure (mm Hg)	75±11	82±9#	79±12
HbA_{1c} (%)	5.7±0.5	6.0±0.5	7.2±1.3**
Fasting glucose (mmol/l)	5.4±0.3	6.3±0.7*	7.0±3.0**
Glucose 120 min (mmol/l)	5.1±1.2	9.0±0.9*	14.4±3.9**
Fasting serum insulin (mU/l)	9.9 (5.3-22.1)	17.4 (5.8-41.5)*	20 (5.8-55.5)*
Serum insulin 120 min (mU/l)	55 (10.5-189.6)	191.4 (28.3-1874)*	194.2 (17.0-532.7)*
Total cholesterol (mmol/l)	6.6±1.2	6.6±1.1	6.4±1.1
HDL-cholesterol (mmol/l)	1.3±0.4	1.3±0.3	1.3±0.3
Triglycerides (mmol/l)	1.4 (0.8-11.3)	1.4 (0.7-3.6)	1.4 (0.6-3.0)
Albuminuria (mg/day)	5.4 (1.3-261)	6.5 (1.4-105)	7.3 (2.1-685)
Retinopathy	none	1M : 2F	2M : 2F
PAD	1M : 4F	2M : 4F	5M : 3F
Angina pectoris	none	1M : 3F	2M : 1F
Myocardial infarction	none	1F	4M : 1F

Data are in means ±SD or medians (ranges). * $p<0.001$ vs normals; ** $p<0.001$ vs normal and IGT; # $p<0.01$ vs normal. PAD: peripheral artery disease.

Fasting blood samples for this cross-sectional survey were collected in tubes containing sodium citrate 3.25% (dilution 1:10) for determining TFPI activity, vWF antigen, thrombomodulin, t-PA antigen and PAI-1 activity, glycated hemoglobin (HbA_{1c}) and lipid profile at baseline.

TFPI activity was measured using the chromogenic substrate assay according to Sandset after polybrene pretreatment (expressed in % with regard to standardized TFPI activity, measured in a plasma pool obtained from 45 healthy donors with a mean age 36±5 years; CV 7.4% at 100% level in our laboratory). In vivo

thrombin formation was assessed by determination of prothrombin fragments F_{1+2} using ELISA (Enzygnost Behring, Marburg, Germany; intra-assay CV 5-7.5%, interassay CV 6-13%). Plasma levels of vWF antigen were determined by an ELISA method using rabbit anti-human vWF (Dako A/S, Denmark; CV 7%; normal range 60-180%). Thrombomodulin (Diagnostica Stago, France; CV 8%; mean 24 ng ml^{-1}) and t-PA antigen (Innogenetics, Belgium; CV 7%; normal range 1.3-10.4 ng ml^{-1}, mean 4.1±2.4 ng ml^{-1}) were measured by enzyme immunoassay. PAI-1 activity was photometrically measured (Kabi Diagnostica, Sweden; intra-assay CV 6%; normal range 1-20 AU ml^{-1}, median 8 AU ml^{-1}). HbA_{1c} was measured by high-performance liquid chromatography (Diamat, Bio-Rad, USA). Serum total and HDL-cholesterol and triglycerides were determined with enzymatic methods (Unimate 5 and 7, Roche, Basle, Switzerland), and apo A_1 and apo B were measured with immunoturbidimetry (Uni-kit, Roche). An enzymatic hexokinase method (Unimate 5, Roche) was used for measuring serum glucose concentrations. Serum free insulin was measured with a double antibody radioimmunoassay after polyethylene glycol pretreatment (Pharmacia Diagnostics; within-assay CV 3.4-6.1% in the range of 3-50 mU l^{-1}). For determination of urinary albumin an immunoturbidimetric technique was applied (Uni-kit, Roche; interassay CV 3%).

Statistics

All data are expressed as mean ±SD or as median and range when not normally distributed. For comparing more than two groups, analysis of variance (ANOVA) with Student-Newman-Keuls correction for multiple comparisons was used. If data were not normally distributed, the Kruskall-Wallis analysis of variance was applied. Categorical variables were analyzed by the chi-square test. Multivariate logistic regression analyses (based on the maximum-likelihood method) were used to investigate the association of TFPI activity with other endothelium-dependent parameters and cardiovascular risk factors. P-values ≤ 0.05 were considered statistically significant.

Results

There were no statistically significant differences in gender between the NGT, IGT and diabetic group (Table 6.1). The mean age, body mass index, and the systolic blood pressure of the NGT subjects were significantly lower than of the subjects with IGT and diabetes. The diastolic blood pressure was significantly higher in the IGT subjects in comparison with the NGT subjects. Serum insulin levels were

higher in the IGT and diabetic group compared to the NGT group. No differences in serum insulin levels were found between the IGT and diabetic subjects. The lipid profile was similar in the three groups.
Since TFPI activity, t-PA antigen, PAI-1 activity and vWF antigen increase with age, the results were adjusted for age[19,23,24]. Compared to the NGT subjects, vWF antigen, t-PA antigen, and PAI-1 activity were all significantly higher in the IGT and diabetic subjects (Table 6.2). TFPI activity was increased in all groups as compared to the younger healthy volunteers, from which the standardized plasma pool was obtained. The TFPI activity was highest in the diabetic patients, while it was higher in the IGT group in comparison with the NGT group. However, the differences were small and of borderline statistical significance. Thrombomodulin levels showed the same pattern. Albumin excretion rate was not different between the three groups. In total, 17 subjects had microalbuminuria, of which 4 subjects had NGT, 4 subjects IGT and 9 subjects had diabetes. One diabetic subject had macroalbuminuria. Also with regard to the procoagulant state, as measured by the prothrombin F_{1+2} fragments, the three groups did not differ.

Table 6.2 Hemostatic endothelium-dependent factors and albuminuria by catagories of glucose intolerance.

	Normal	IGT	Type 2 diabetes
TFPI-act (%)	111±16	113±15	116±16
vWF (%)	127±30	179±44*	168±63*
t-PA (ng/l)	10.7±3.5	14.0±4.2*	15.4±5.8*
PAI-1 (AU/l)	13 (2-26)	23 (3-53)**	16 (2-60)*
TM	39.7±10.3	44.9±12.8	41.8±15.7
F_{1+2}	1.85 (0.54-9.31)	1.98 (0.49-13.57)	1.47 (0.18-13.57)

Data are in means ±SD or medians (ranges). Test for trend, adjusted for age. * $p<0.001$ vs normal; ** $p<0.01$ vs normal.

When correlating the different hemostatic endothelium-dependent factors with the metabolic parameters, it appeared that TFPI activity, vWF antigen and PAI-1 activity were significantly positively correlated with HbA_{1c} (Table 6.3). These correlations however were moderate. There was a correlation between fasting blood glucose and vWF antigen, t-PA antigen and PAI-1 activity, while serum insulin was strongly correlated with vWF antigen ($r=0.34$), t-PA antigen ($r=0.60$) and PAI-1 activity ($r=0.47$). Total cholesterol was only correlated with PAI-1 activity. In addition, there was a negative correlation between HDL-cholesterol and t-PA antigen, PAI-1 activity and thrombomodulin. PAI-1 activity and t-PA antigen were positively associated with triglycerides. The partial correlation coefficients between the various endothelial-dependent factors and cardiovascular risk factors are

depicted in Table 6.4. Besides TFPI activity, vWF antigen and PAI-1 activity were positively correlated with the urine albumin excretion. Table 6.5 shows the interrelationships between the endothelium-dependent factors. PAI-1 activity and thrombomodulin were associated with vWF antigen and t-PA antigen, while the latter was also correlated with vWF antigen. There appeared to be a borderline correlation between TFPI activity and vWF antigen and PAI-1 activity.

Table 6.3 Correlations between endothelium-dependent factors and metabolic parameters.

	TFPI	vWF	t-PA	PAI-1	TM	F_{1+2}
HbA_{1c}	0.18*	0.20*	0.17#	0.16*	-0.11	-0.11
Glucose	0.15#	0.23**	0.25**	0.32***	-0.09	-0.09
Insulin	0.11	0.34***	0.60***	0.47***	0.05	-0.06
Total cholesterol	0.04	-0.10	0.01	0.16*	-0.12	0.03
HDL-cholesterol	-.001	-0.12	-0.33***	-0.22**	-0.16*	-0.07
Triglycerides	0.07	0.02	0.21*	0.30***	-0.03	-0.04

Values are partial coefficients, adjusted for age. # $p<0.10$; * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Table 6.4 Correlations between endothelium-dependent factors and cardiovascular risk factors.

	TFPI	vWF	t-PA	PAI-1	TM	F_{1+2}
Systolic blood pressure	0.05	0.02	0.08	0.17*	0.11	0.10
Diastolic blood pressure	0.03	0.01	0.04	0.17*	0.01	0.19*
Albuminuria	0.16*	0.21*	0.16#	0.22**	0.13	-0.02
Body Mass Index	0.04	0.20*	0.42***	0.45***	0.09	0.13

Values are partial correlation coefficients, adjusted for age. # $p<0.10$; * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Table 6.5 Correlations between endothelium-dependent factors.

	TFPI	vWF	t-PA	PAI-1	TM
vWF	0.14#				
t-PA	0.02	0.42***			
PAI-1	0.14#	0.25**	0.48***		
TM	-0.05	0.22**	0.18*	-0.04	
F_{1+2}	-0.002	-0.03	0.03	-0.09	0.10

Values are partial correlation coefficients, adjusted for age. # $p<0.10$; * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Discussion

Atherosclerotic changes of the arterial vascular bed is one of the main events of ageing. Disturbances in glucose metabolism accelerate this process of atherosclerosis. Since endothelial damage plays a central role in the pathogenesis of cardiovascular disease, we have investigated several endothelium-dependent hemostatic factors in the present study. One may thereby assume that in subjects with a disturbed glucose metabolism more vascular endothelial dysfunction is present in comparison with age-matched healthy controls. More vascular dysfunction may hereby be reflected by higher levels of circulating endothelium-derived factors.

We observed significantly increased levels of vWF antigen, t-PA antigen, and PAI-1 activity in the IGT group as well as in the diabetic group as compared to the eldery with NGT. This confirms earlier reports, in which it was also suggested that these alterations were the result from damage of endothelial cells[25]. Since there were no differences between the IGT and diabetic group, the increases in levels of vWF antigen, t-PA antigen and PAI-1 activity could suggest that part of the endothelial damage may already occur in the prediabetic phase. This may explain the reported increased incidence of cardiovascular disease in these two groups. It is known that the levels of vWF antigen, t-PA antigen and PAI-1 activity are influenced by glucose metabolism[26-28]. As compared to the NGT group, fasting glucose was highest in the IGT and diabetic group, while glycated hemoglobin was significantly higher in the type 2 diabetic subjects. Correlation analysis indeed not only showed a positive association between HbA_{1c} and vWF antigen, but between fasting blood glucose and vWF antigen, t-PA antigen and PAI-1 activity as well. In addition, serum insulin correlated also with these three endothelial hemostatic factors. Especially, there was a strong relation between insulin levels and PAI-1 activity and t-PA antigen, which remained after further adjustment for serum glucose and HbA_{1c}. These findings suggest that higher insulin levels, and therefore probably insulin resistance, are associated with impaired fibrinolysis in elderly subjects with or without diabetes mellitus.

Thrombomodulin levels were increased in the three groups, but no differences between the groups could be demonstrated. However, thrombomodulin did not correlate with the risk factors or any of the metabolic parameters with the exception of HDL-cholesterol. This is in contrast with earlier reports[15].

Although we found a slightly higher TFPI activity in the IGT and diabetes groups, this difference was not statistically significant. However, we were able to demonstrate a significant association between TFPI activity and HbA_{1c}. This is in agreement with the findings of Kario et al, who also found increased TFPI antigen levels in type 2 diabetic patients, especially with overt albuminuria, compared to

healthy controls[17]. In the present study, a significant correlation between TFPI activity and the albumin excretion rate could also be demonstrated. Since microalbuminuria is a sign of generalized angiopathy, TFPI may be a marker of this condition. The same correlation could also be demonstrated between albuminuria and vWF antigen and PAI-1 activity. However, in comparison with type 1 diabetes, type 2 diabetes is considered to be a different disease regarding vascular complications. While type 2 diabetes is characterized by the occurrence of macrovascular complications, in type 1 diabetic patients mainly microvascular complications are observed before macrovascular abnormalities occur. TFPI expression is restricted to the endothelium of the microvasculature and is not believed to be synthesized by the endothelium of larger vessels[29]. This may partly explain why in the present study no significant differences between the diabetic and NGT group are found. In addition, the elderly subjects in the present study population appeared to be relatively free of cardiovascular disease. Besides, only a total of 17 subjects in all three groups had microalbuminuria, while 1 subject with diabetes had macroalbuminuria. The presence of extensively generalized angiopathy would therefore be less likely. Others found increased levels of TFPI only in type 2 diabetic patients with overt albuminuria[17].

It is widely accepted that in patients with diabetes, a procoagulant state can be found[30,31]. Because TFPI is a coagulation inhibitor, one may suggest that TFPI activity is influenced by this procoagulant state. In the present study, we were unable to show a difference in procoagulation, as measured by the prothrombin F_{1+2} fragments, between the three groups. There was also no significant correlation between TFPI activity and the F_{1+2} fragments in this patient population. Others were also unable to demonstrate a correlation between TFPI and factor VIIa levels[17]. In addition, we could not find a relation between F_{1+2} and the other endothelial hemostatic factors.

Dyslipidemia is a well recognized risk factor for cardiovascular disease in IGT and type 2 diabetic subjects[4,6]. Because TFPI in plasma is mainly associated with lipoproteins, especially with LDL and HDL[20,21], TFPI activity could be influenced by the levels of lipoproteins. The levels of lipoproteins were similar in the three groups, and no significant relationship between TFPI activity and lipids was found. This is in agreement with our earlier report on type 1 diabetic patients, while others have found a positive relation in nondiabetic hypercholesterolemic subjects[16,32]. Only PAI-1 activity was associated with total cholesterol, while there was a negative correlation between HDL-cholesterol and t-PA antigen, PAI-1 activity and thrombomodulin.

The endothelium-dependent factors are also known to be affected by blood pressure[33-35]. The systolic blood pressure was higher in the IGT and diabetic subjects, while the diastolic blood pressure was higher in the IGT group. However, the systolic and diastolic blood pressure were only positively correlated with PAI-1

activity. Obesity is another well known risk factor for cardiovascular complications[36]. The BMI was higher in the IGT and diabetic group. In addition, a significant positive correlation between BMI and t-PA antigen, PAI-1 activity and vWF antigen could be demonstrated. This is in concordance with the findings of earlier studies[37-39].

We conclude that several hemostatic endothelium-dependent factors are already increased in elderly subjects, relatively free of cardiovascular disease, with impaired glucose tolerance, which is believed to precede the development of type 2 diabetes mellitus. This seems to be especially true for vWF antigen, t-PA antigen and PAI-1 activity. TFPI activity and thrombomodulin levels were increased in all elderly subjects with a normal or impaired glucose tolerance and type 2 diabetes mellitus, but there were no differences between the groups.

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Chapter 7

Tissue Factor Pathway Inhibitor (TFPI) release from cultured endothelial cells under normo- and hyperglycemic conditions

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Abstract

It has recently been demonstrated that basal and postheparin TFPI activity is increased in patients with diabetes mellitus. In order to find an explanation for these in vivo observations, an in vitro study was performed, using an immortalized human endothelial cell (EC) line (EA.hy 926). After the cells were cultured in the presence of varying concentrations of D-glucose and insulin, the cells were incubated for 10 minutes with unfractionated heparin. Then, TFPI activity was measured in the supernatant and at the EC surface. Postheparin TFPI activity in the supernatant increased with increasing heparin concentrations up to 0.6 U/ml. The time course of TFPI release using 0.5 U/ml heparin showed a rapid release during the first 10 minutes. Interestingly, the cell-bound TFPI activity decreased as expected during that time period, but not further than 35%, in spite of prolonged incubation time. Postheparin TFPI release, but not basal TFPI activity, was higher in EC cultures with 35 mmol/l D-glucose than from EC cultured with 7 mmol/l D-glucose ($p<0.001$). This difference was not due to a change in osmolarity or number of cells. Postheparin TFPI activity in EC cultures was positively correlated with glucose ($r=0.28$, $p<0.05$). In high glucose EC cultures with low insulin concentrations postheparin TFPI activity was increased, while high insulin concentrations had the opposite effect. In low glucose EC cultures, insulin had no effect on postheparin TFPI activity. High glucose levels may be an explanation for these findings since they could cause glycation of glycosaminoglycans at the endothelial cell surface, which in turn could result in altered binding characteristics of TFPI to these glycosaminoglycans.

Introduction

The vascular endothelium can be regarded as a paracrine organ which regulates hemostasis (coagulation and fibrinolysis). The fact that blood does not form clots continuously as it passes over the endothelial cell surface indicates that in homeostasis anticoagulant mechanisms predominate over procoagulant processes[1]. The endothelium expresses factors associated with anticoagulant properties such as heparan sulfate, thrombomodulin and tissue factor pathway inhibitor (TFPI)[1-3]. Besides, it can directly influence vascular fibrinolysis through the synthesis of fibrinolytic components that promote or inhibit fibrinolysis (respectively tissue plasminogen activator (t-PA) and plasminogen activator inhibitor-1 (PAI-1))[1].

In 1987 TFPI, a Kunitz-type coagulation inhibitor, was indentified as a single protein[4,5]. The TFPI gene has been localized on chromosome 2q[6]. TFPI inhibits factor Xa and in complex with factor Xa also the factor VIIa/tissue factor catalytic complex. The major site of production is the vascular endothelium[3]. Of TFPI 50-90% is bound to the endothelium, probably by glycosaminoglycans, 10-50% in plasma and 2-3% in platelets[7]. Only about 5% of the TFPI in the plasma circulates as a free, uncomplexed protein[8]. Release from the endothelial cell surface can be stimulated by glycosaminoglycans with greater binding affinity to TFPI like heparin[9].

Diabetes mellitus is associated with premature atherosclerosis. Endothelial damage has thereby been considered as the initiating event in the pathogenesis of atherosclerosis[10,11]. Disturbances in hemostatic parameters in diabetic patients play a role in the development of cardiovascular complications. Until now little is known about TFPI activity in diabetic subjects. Previously, we have performed a study concerning TFPI activity in the basal state and after intravenous administration of heparin in IDDM patients in comparison to healthy controls[12]. An increased basal and postheparin TFPI activity was found in the IDDM patients as compared to the controls, related to poor metabolic control. This increase in TFPI activity may be the result of functional vascular endothelial damage[13]. An increased TFPI activity as a counteraction to the procoagulant state seen in diabetic patients could not be demonstrated[13]. However, altered binding characteristics of TFPI to glycosaminoglycans at the endothelial cell surface, due to chronic hyperglycemia, could play a role.

Because hyperglycemia and hyperinsulinemia are found in diabetic patients, we performed several in vitro studies to evaluate TFPI activity in cultures of human endothelial cells under different in vitro conditions, comparable with different metabolic conditions as can be seen in diabetic patients. TFPI activity was not only measured in the supernatant but also indirectly at the endothelial cell surface as well. A human endothelial hybrid cell line, EA.hy 926, was used, which has

already been evaluated regarding the expression of TFPI and other endothelium-dependent parameters, such as von Willebrand factor, thrombomodulin, t-PA and PAI-1[14-16].

Material and Methods

Materials

The culture medium RPMI 1640 (code 42402) and Medium 199 (code 41150) were purchased from Gibco, the 24 Falcon multiwell dishes (2 cm^2/well) from Micronic. The polyclonal rabbit anti-human TFPI antibodies were kindly provided by O Nordfang, Novo Nordisk, Denmark. The bovine factor X, D-glucose, sorbitol and insulin were obtained from Sigma, and human thromboplastin from Behring. The chromogenic substrate S2765 was purchased from Chromogenix, the unfractionated heparin from Leo.

Cell cultures

An immortalized human endothelial cell (EC) line (EA.hy 926; kindly provided by CJS Edgell, USA) was used. A mixture of 20 ml RPMI 1640 (20 mM Hepes, 10 mM bicarbonate) and 20 ml Medium 199 (26 mM bicarbonate, 148 mM L-alanyl glutamine) was used as a primary medium, to which 6 ml of fetal calf serum, 1 ml of HAT (mixture of 100 mmol/ml hypoxanthine, 0.4 mmol/ml aminopterine and 16 mmol/ml thymidine), 1 ml of L-alanyl glutamine (200 mM), and 0.5 ml of a mixture of penicillin (10.000 U/ml), streptomycin (10.000 U/ml) and fungizone (25 U/ml) was added. Before use, the complete culture medium was filtered by a sterilizing filter (Minisart, Satorius; pore-width 0.20 μ).

The experiments were performed on confluent EC layers, cultured in 24 multiwell dishes (2 cm^2/well). In a 5% CO_2 stove, cells were cultured at 37°C in wells containing 0.5 ml of medium. The endothelial cells were seeded in such a density that confluency was not reached earlier than after 5 days. When not stated otherwise, the cell culture medium was not refreshed during culturing.

Cell counts

The cell cultures at confluency were washed twice with phosphate-buffered saline (PBS). Thereafter, the cells were harvested by incubation with 0.05% trypsin EDTA 0.02% (ICN). The trypsin was removed after centrifugation (1200 RPM, 5 min), while the cell pellet was resuspended in primary medium solution. Cell

counts of cultures were then performed, using a cell count chamber (Bürker-Turk; Optik-Labor).

Measurements of TFPI activity

TFPI activity in the supernatant was measured chromogenically according to Sandset[17]. TFPI activity was also measured at the EC surface. Confluent cell layers, cultured in 10 wells of a 24 multiwell dish (2 cm^2/well), were rinsed with Hepes-buffer (Hepes 10 mmol/l, NaCl 135 mmol/l, KCl 4 mmol/l, $CaCl_2$ 3 mmol/l, Mg_2Cl 1 mmol/l, D-glucose 11 mmol/l, bovine serum albumin (BSA) 25 mg/ml, pH 7.45) twice. Then each well was incubated with 100 μl Hepes-buffer for 10 min at room temperature (step 1). In a control experiment, instead of Hepes-buffer, 10 μl polyclonal rabbit anti-human TFPI antibodies in Hepes-buffer (concentration 1:200) was incubated for 10 min under the same conditions. After incubation Hepes-buffer and anti-TFPI were removed from the cell layers, 40 μl of a mixture containing 50 mmol/l Tris.HCl, 0.1% BSA, 10 mmol/l calcium, 2.5 nmol/l bovine factor X, 2 nmol/l factor VIIa, and human thromboplastin (1:300) was added to all the wells except one, which also served as a control (step 2). To this well, Hepes-buffer was added. Subsamples (25 μl) were taken after variable incubation times (1 to 25 min) to measure residual factor VIIa/tissue factor activity in the supernatant. The multiwell dish was carefully shaken during the subsampling periode, using a thermostated microwell shaker. To the subsamples 65 μl bovine factor X (70 nmol/l) was added and incubated for 15 min at 37°C (step 3). Hereafter, 50 μl chromogenic substrate S2765 (0.8 mg/ml) was added to the subsamples and incubated for 20 min at 37°C (step 4). The conversion of substrate was stopped with 50 μl of 20% acetic acid and read off at 405 nm. The measurement of TFPI activity at the endothelial cell surface was also performed after incubation with 0.5 U/ml heparin (step 1, instead of Hepes-buffer) for 10 min at 37°C.

Osmolarity experiment

Osmolarity studies were done, using D-glucose and sorbitol (30 and 60 mmol/l). Osmolarity was measured by the freezing-point depression method (Fiske 2400 multisample osmometer, Fiske Associates, Norwood, USA). Glucose was measured by an enzymatic hexokinase method (Unimate 5, Roche).

Heparin incubation experiments

After rinsing the confluent EC layer twice with Hepes-buffer, the cell culture was incubated with 200 μl(0.5 U/ml) of unfractionated heparin (Leo) in Hepes-buffer during 10 minutes at 37°C, unless stated otherwise.

Statistics

All data are expressed as mean ±SD. For comparing two groups the t-test was applied, while in case of comparing more than two groups, one-way analysis of variance (ANOVA) with Student-Newman-Keuls correction for multiple comparisons was used. In case of repeated measurements in time a multiple analysis of variance (MANOVA) for repeated measurements was performed. P-values ≤ 0.05 were considered statistically significant. The correlation coefficient was also determined by regression analysis.

Results

Heparin-dependent TFPI release from cultured endothelial cells

To establish the time-dependency of TFPI release, day 1 confluent EC layers were rinsed with Hepes-buffer and incubated with heparin (0.5 U/ml) for variable time intervals (1 to 30 minutes; n=12, Figure 7.1A). It is apparent that TFPI activity increased rapidly during the first 10 minutes. After that a much slower release was observed. Therefore, we also indirectly measured the TFPI activity at the cell surface for several time intervals (1 to 25 minutes) with and without heparin. Figure 7.1B clearly shows a rapid inactivation of the factor VIIa/tissue factor complex, resulting in a decrease of residual activity in the supernatant to about 35%. After heparin incubation, this decrease in residual factor VIIa/tissue factor activity in the supernatant appeared to be somewhat less, suggesting that cell-bound TFPI can only be partially displaced by heparin under the conditions of the experiment. The specificity of this experiment was demonstrated by adding anti-human TFPI antibodies to the EC layers, resulting in no change of factor VIIa/tissue factor activity in the supernatant. The time course of cell-bound residual TFPI activity appeared to be complementary with the time course of heparin releasable TFPI shown in Figure 7.1A.

Next, the optimal concentration of unfractionated heparin for TFPI release was investigated. After rinsing the confluent EC layer twice with Hepes-buffer, the cells were incubated with unfractionated heparin (200 μl) of different concentrations (0 to 1.6 U/ml) during 10 minutes at 37°C. Figure 7.2 shows that heparin concentrations higher than 0.6 U/ml did not result in a further significantly higher TFPI release (n=6).

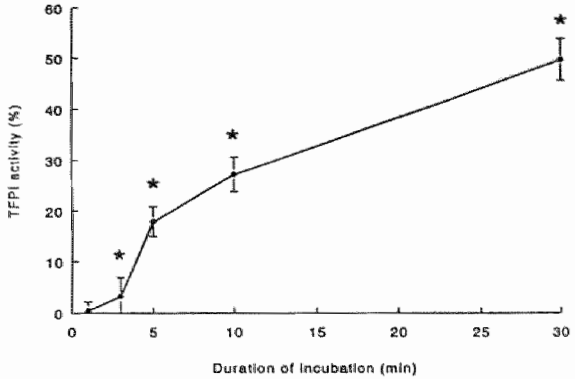

Figure 7.1A Time-dependency of heparin-induced TFPI release. The influence of the duration of incubation with 0.5 U/ml heparin on TFPI release in cultured EC was studied by measuring TFPI activity in the supernatant after heparin incubation time intervals varying from 1 to 30 minutes. * $p < 0.0001$ as compared to preceding measurement.

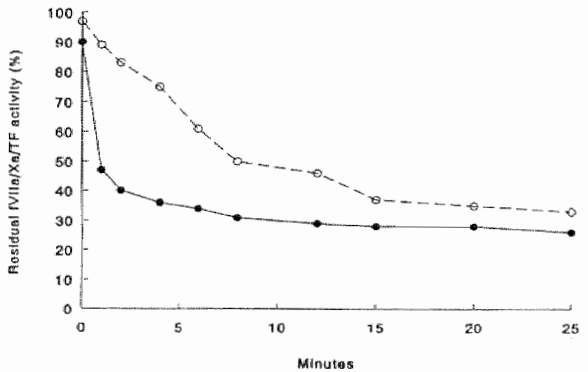

Figure 7.1B Time-dependency of the decrease in residual factor VIIa/Xa/TF activity in the supernatant before and after heparin incubation. The cell-bound TFPI activity was measured indirectly by measuring residual factor VIIa/Xa/TF activity in the supernatant before (•) and after (o) heparin incubation. Confluent cell layers were incubated with Hepes-buffer for 10 min at room temperature (step 1). After removal of Hepes-buffer, a mixture of Tris.HCl, BSA, calcium, bovine factor X (2.5 nmol/l), factor VIIa and human thromboplastin was added (step 2). Thereafter, subsamples were taken after 1 to 25 minutes of incubation to measure residual factor VIIa/tissue factor activity. To these subsamples bovine factor X (70 nmol/l) was added and incubated for 15 min at 37°C (step 3). Finally, chromogenic substrate S2765 was added to the subsamples and incubated for 20 min at 37°C. Conversion of substrate was stopped and read off at 405 nm. The procedure was also performed after incubation with 0.5 U/ml heparin (step 1, instead of Hepes-buffer).

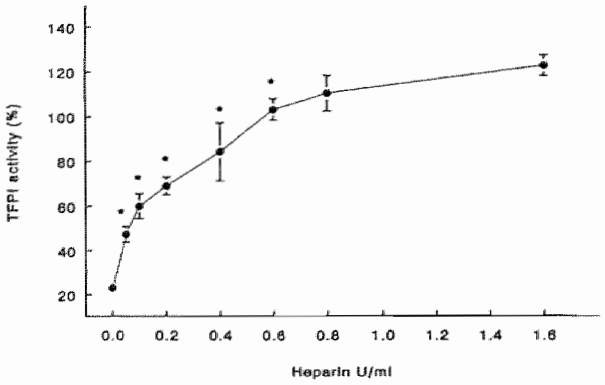

Figure 7.2 Heparin concentration and TFPI release. Influence of various heparin concentrations (0 to 1.6 U/ml) on TFPI activity in EC cultures. Duration of incubation with heparin: 10 min at 37°C. * $p<0.05$ vs preceding measurement.

Another variable that could determine the amount of TFPI that can be released by heparin from cultured endothelial cells is the 'culture-time'. Before heparin incubation, basal TFPI activity in the medium was significantly higher on day 4 postconfluent as compared to day 1 ($n=12$; $1561\pm139\%$ vs $1229\pm65\%$, $p<0.0001$). The amount of TFPI that could be released from rinsed endothelial cells by heparin (0.5 U/ml; incubation time 10 minutes) was significantly higher for cells that were already confluent for 4 days as compared to cells that were only 1 day confluent ($n=9$; $172\pm30\%$ vs $99\pm14\%$, $p<0.0001$). A significant correlation between pre- and postheparin TFPI activity ($r=0.54$, $p<0.0001$) could also be demonstrated.

To see whether the postheparin TFPI activity is dependent on TFPI concentration in the medium, varying TFPI activities in the culture medium were obtained by adding nothing, fresh medium (0.5 ml/well) or 'old' (pre-grown) medium (0.5 ml/well) at day 3 of culture (Figure 7.3). Old medium was obtained by removing the supernatant of 3 days old cell cultures. When the TFPI concentration in the supernatant was changed by adding fresh medium on day 3 of culture, significantly lower postheparin TFPI activity were found as compared to TFPI activity in medium that was not refreshed ($n=9$; $98\pm6\%$ vs $124\pm13\%$, $p<0.001$) or in medium to which old medium was added ($n=9$; $98\pm6\%$ vs $114\pm10\%$, $p<0.01$). No difference was seen between no change of medium and the addition of old medium.

From the results of these initial experiments the following conditions were defined for further experimental work. Endothelial cells, one day after confluency was reached, were rinsed twice with Hepes-buffer prior to incubation with Hepes-buffer containing 0.5 U/ml unfractionated heparin during 10 minutes at 37°C. During

culturing, no replacement or changing of the medium was allowed. Only when stated otherwise, all in vitro studies concerning the effects of glucose and insulin on TFPI activity were performed under these conditions.

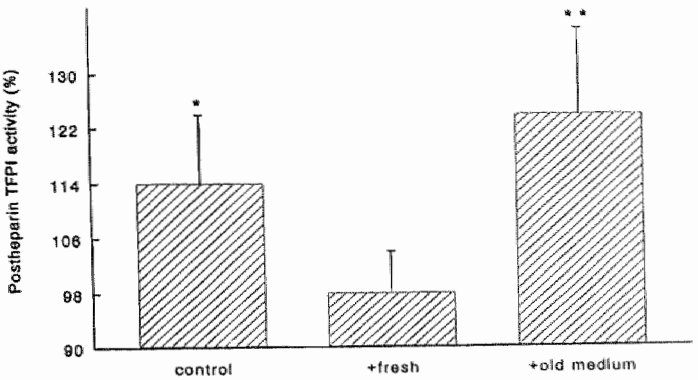

Figure 7.3 Influence of preheparin TFPI activity on postheparin TFPI release. The effect of different TFPI concentrations in the culture medium on postheparin TFPI activity is shown in this figure. The medium was not changed (*control*) or either old (*+old medium*) or fresh medium (*+fresh*) was added on day 3 of culture. * $p<0.01$ vs *+fresh*, ** $p<0.001$ vs *+fresh*.

Effects of D-glucose and insulin on TFPI activity

Different concentrations of D-glucose (7 and 35 mmol/l) were added to culture medium to study the effects of D-glucose on TFPI activity. Before heparin incubation, no difference in basal TFPI activity in the supernatant between low and high glucose cultures was seen ($n=12$; $1229\pm65\%$ vs $1109\pm47\%$, NS). However, when rinsed cells were incubated with various heparin concentrations higher TFPI activities were recovered from cells that were grown in medium with high glucose concentration as compared to cells with low glucose culture conditions (Figure 7.4).

High glucose concentrations in the culture medium meant significantly higher postheparin TFPI activity on day 1 as well as on day 4 postconfluent ($n=12$; day 1: $99\pm14\%$ vs $138\pm21\%$, $p<0.0001$; day 4: $172\pm30\%$ vs $203\pm23\%$, $p<0.05$). A positive correlation between postheparin TFPI activity and glucose was found ($r=0.28$, $p<0.05$).

Cell counts were performed on the first day of confluency. No difference in cell counts could be demonstrated between cell cultures with a low and high glucose medium ($n=24$; $20.3\pm7.6 \times 10^6$ cells/ml versus $21.8\pm7.0 \times 10^6$ cells/ml, NS). An osmolarity study was performed using sorbitol; a culture without adding D-glucose

or sorbitol served as a control. While cell cultures with D-glucose showed a significant increase of postheparin TFPI activity (n=6; at 30 mmol/l: 91±6%, at 60 mmol/l: 88±6% vs control 72±13%, $p<0.05$), no increase was seen with equal sorbitol concentrations in the culture medium.

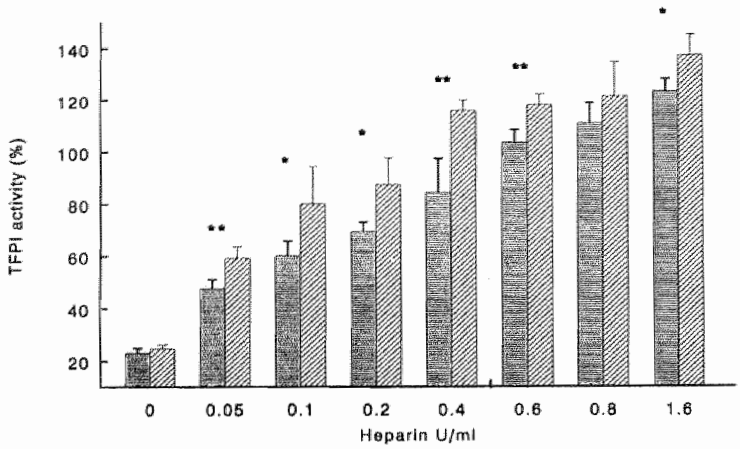

Figure 7.4 Effect of low and high glucose culture medium on TFPI activity. After incubation with various heparin concentrations TFPI release is measured in cultures of EC cultured in high glucose medium (35 mmol/l D-glucose; hatched bars) and in low glucose medium (7 mmol/l D-glucose; black bars). * $p<0.01$, ** $p<0.001$.

When studying the effects of different insulin concentrations (0 to 65 mU/l) in low and high glucose cell cultures, low insulin resulted in a higher postheparin TFPI activity in a high glucose culture (Figure 7.5, n=8; 121±3% vs 110±6%, $p<0.05$). On the other hand, high insulin concentrations in high glucose cell cultures led to a decrease in postheparin TFPI activity, equal as could be found in the low glucose cell cultures. As a control experiment, insulin solvent (acetic acid) alone did not influence TFPI activity. In low glucose cell cultures, no evident effect of insulin on postheparin TFPI activity was seen. Before heparin incubation, no differences in basal TFPI activity in the supernatants of the various cell cultures were observed.

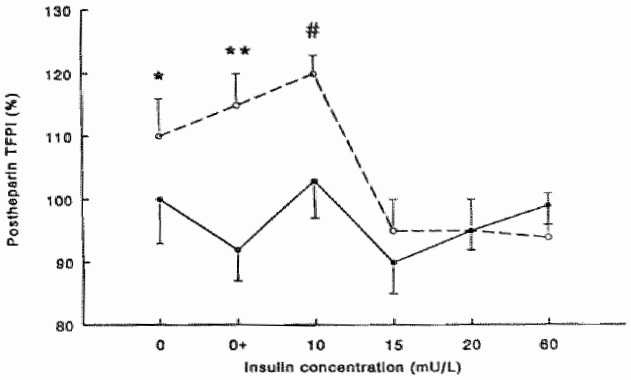

Figure 7.5 Influence of insulin on postheparin TFPI release in relation to low and high glucose concentrations in culture medium. The effect of various concentrations of insulin (0 to 60 mU/l) in medium with low and high glucose on postheparin TFPI. • EC cultured in medium with 7 mmol/l glucose, ○ EC cultured in medium with 35 mmol/l glucose, 0+ insulin solvent (acetic acid); * <0.05, ** $p<0.001$ vs insulin concentrations of 15, 20, and 60 mU/l; # $p<0.05$ vs insulin concentration of 0 mU/l, $p<0.001$ vs insulin concentrations of 15, 20, and 60 mU/l.

Discussion

The present study demonstrates that heparin induces dosage and time dependently release of TFPI from cultured EC with a concomitantly decrease in EC-bound TFPI activity. D-glucose increases heparin-induced TFPI release from cultured EC, independently of the concentration of heparin, duration of confluence of the EC layers, and osmolarity. Insulin only affects postheparin TFPI release in high glucose EC cultures.

The release of TFPI from EC by heparin appeared to be dependent from the concentration of heparin, with a maximal TFPI release when the heparin concentration was between 0.4 and 0.6 U/ml during an incubation period of 10 minutes. This finding suggests a binding of TFPI to the cell surface in a saturable manner, which is supported by in vivo and in vitro studies[18,19]. In addition, a longer duration of incubation with heparin resulted in an increased TFPI release up till 30 minutes. However, a flattening of the TFPI release curve is observed after 10 minutes of incubation. Evidence has recently been provided for the existence of TFPI-specific glycosyl phosphatidylinositol (GPI)-anchored binding sites on external membranes[20]. There is support that this is also true for EC[21]. One could hypothesize that there exist heparin-unreleasable TFPI at GPI-anchored binding sites and heparin-releasable glycosaminoglycan-bound TFPI, both located at the

endothelial cell surface. Our finding that cell-bound TFPI can only partially be released by heparin is compatible with this notion. Recently, an increased secretion and redistribution of cellular TFPI induced by heparin in EC cultures has been demonstrated[22].

It appeared that TFPI release by heparin also depends upon TFPI activity in the culture medium during culturing. Lowering the TFPI activity in the culture medium by adding fresh medium during culturing resulted in a decreased TFPI release after heparin incubation. We were also able to show a significant positive correlation between pre- and postheparin TFPI activity. Apparently, there is an equilibrium between 'free' TFPI in the medium and endothelial cell-bound TFPI. In vivo, this is supported by the study of Kokawa et al., who found a positive correlation between free TFPI and endothelial cell-associated TFPI in hyperlipidemic patients[23].

Heparin released more TFPI from cells that were cultured in high glucose medium than from cells cultured in low glucose medium. This higher release was not caused by osmotic mechanisms or increased number of cells. This in vitro finding might provide a basis for our earlier observation of increased postheparin TFPI activity in type 1 diabetic patients[12]. A number of studies have demonstrated that high glucose in endothelial cell cultures results in increased levels of other endothelium-dependent hemostatic parameters like von Willebrand factor, t-PA and PAI-1[24,25]. It is our notion that chronic high glucose levels affects the endothelial function in vitro as in vivo, resulting in increased TFPI release after heparin.

In diabetes, function and/or structure of endothelial glycosaminoglycans, could be altered. Animal studies suggest that in diabetes glycosaminoglycans are less negatively charged, due to the synthesis of low-sulfated heparan sulfate molecules[26,27]. TFPI is most likely bound to glycosaminoglycans with its highly positively charged COOH-terminus[28]. We hypothesize that the increased TFPI release after heparin incubation of endothelial cell cultures with high glucose may therefore be explained by a weaker binding. This is supported by the fact that we were unable to find differences in basal (preheparin) TFPI activity in the supernatants of EC cultured with low and high glucose medium. Alterations in function of glycosaminoglycans could be the result of nonenzymatic glycation, formation of advanced glycated endproducts and oxidative stress, as can be found in diabetic patients[29-31]. It has been shown that some effects of high glucose in endothelial cell cultures can be reversed by antioxidants[32].

In the present study, low insulin levels resulted in higher TFPI release after heparin incubation only in the high glucose cultures, while high insulin levels in high glucose cultures led to decreased postheparin TFPI release. No effect of insulin was seen in the low glucose cell cultures. Abe et al. also found an effect of insulin only in endothelial cell cultures with high glucose[33]. In agreement with our results,

an inverse correlation between heparin-stimulated TFPI antigen plasma levels and plasma insulin concentrations has recently been reported in man[34].

In conclusion, heparin-stimulated release in EC cultures is parallelled by a decrease of TFPI activity at the EC surface. High D-glucose concentrations (in combination with low insulin concentrations) in EC cultures increases TFPI release by heparin.

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Chapter 8

General discussion and conclusions

General discussion and conclusions

As long as there is no cure for diabetes mellitus, treatment is focused on attaining the best possible metabolic control to prevent the development of secondary complications on the short and long term. In 1993 the results of the Diabetes Control and Complication Trial indicated that a good metabolic control reduces the risk of developing microvascular complications in type 1 diabetic patients[1]. We therefore need markers which can identify those patients who are at risk for developing micro- and macrovascular complications in an early stage of their disease. Until now, von Willebrand factor (vWF) appears to be such a marker[2]. Because of its hemostatic endothelium-dependent characteristics we hypothesized that TFPI may also be a valuable marker of endothelial (dys)function in diabetic subjects. Not only did we find significantly higher pre- and postheparin TFPI activities in plasma of patients with type 1 diabetes mellitus without complications in comparison with healthy volunteers, but a strong positive correlation between TFPI activity and glycated hemoglobin as well (*Chapter 3*). This finding indicates that TFPI activity is affected by chronic hyperglycemia. Because of the fact that TFPI is mainly produced by and bound to the vascular endothelial cells, one may speculate that the increased TFPI activity in plasma of this group of type 1 diabetic subjects without clinically detectable (vascular) complications reflects early endothelial damage or dysfunction. If this is true, additional therapeutic interventions should be considered in these patients, especially with a poor metabolic control. Very recently it is demonstrated that optimisation of glycemic control in poorly controlled type 1 diabetic patients induces normalisation of TFPI activity[3].

In the pathogenesis of atherosclerosis endothelial damage is an early feature. This is followed by a cascade of events (of which one important event is the activation of the tissue factor pathway), which eventually results in the development of the atherosclerotic plaque. Microvascular complications, especially microalbuminuria, are associated with an increased risk of developing cardiovascular diseases in diabetic and non-diabetic subjects[4,5]. It is thereby generally accepted that microalbuminuria reflects generalized angiopathy[6]. Alterations in synthesis and/or function and/or structure of glycosaminoglycans in diabetes could form the pathophysiological basis of microalbuminuria[7]. In type 1 diabetic subjects with microalbuminuria we found significantly higher basal and postheparin TFPI activities as compared to diabetic subjects with no complications or with only retinopathy (*Chapter 4*), while the overall metabolic control in the three groups was equal. In addition, a strong correlation between basal TFPI activity and microalbuminuria could be demonstrated. While TFPI is most likely bound to the endothelial cell surface by glycosaminoglycans we hypothesize that the increased TFPI activity in type 1 diabetic subjects with microalbuminuria may be the result

of altered endothelial glycosaminoglycan characteristics.

On the other hand, increased TFPI activity could also be triggered by a procoagulant state as can be found in diabetes mellitus. In patients with type 1 diabetes mellitus and microalbuminuria, increased TFPI activity and levels of prothrombin F_{1+2} fragments were seen (*Chapter 5*). However, we found no correlation between the two variables, suggesting that the increase in TFPI activity in plasma is more complex than just a reflection of a procoagulant state.

Recently, we learned from the results of the UK Prospective Diabetes Study that improved metabolic control also leads to a decrease in the risk of microangiopathy in patients with type 2 diabetes mellitus[8]. In our population-based study, TFPI activity was increased in elderly subjects with a normal or impaired glucose tolerance (a state which precedes the development of type 2 diabetes mellitus) and type 2 diabetes mellitus (*Chapter 6*). However, there were no significant differences in activity seen between the three groups. One must take into account that TFPI activity was measured chromogenically and compared with a standard curve obtained from a pool of 45 healthy donors, with a mean age of 36 $\pm$ 5 years. It is known that TFPI activity is increasing with age. From these data, it may be concluded that TFPI probably can not be used as a marker of endothelial dysfunction to discriminate groups at risk in an elderly population.

In cultured endothelial cells, derived from an immortalized endothelial cell line (EA.hy 926), we were able to demonstrate that high glucose conditions, as in vivo, not only induce increased TFPI activity in the medium but also leads to an increased release of TFPI from the endothelial cell surface after heparin (*Chapter 7*). A positive correlation between postheparin TFPI activity and D-glucose concentration of the culture medium was found. Insulin only affected postheparin TFPI release in high glucose EC cultures. As mentioned earlier, diabetes is characterized by alterations in glycosaminoglycans[7]. A possible explanation for these findings may therefore be glycation of glycosaminoglycans at the endothelial cell surface by high glucose levels, which in turn could result in an altered binding of TFPI to these glycosaminoglycans.

In conclusion, basal TFPI activity and TFPI release from endothelial cells after heparin is increased in vivo and in vitro by chronic hyperglycemic conditions. Although not specifically investigated, we hypothesize that chronic hyperglycemia may lead to alterations in metabolism and/or functional structure of glycosaminoglycans, resulting in altered binding characteristics between TFPI molecules and glycosaminoglycans at the endothelial cell surface. These changes may eventually play a role in the occurrence of cardiovascular events in patients with diabetes mellitus.

Could TFPI activity in plasma be the common denominator for some important factors (such as endothelial damage, resulting in a decreased anticoagulant potential

at the surface of the endothelium, thus increasing the risk of thrombin formation on damaged endothelium) leading to the development of microvascular and cardiovascular complications in diabetes ? To answer this question, further (prospective) clinical trials and in vitro studies are needed. However, more reports of increased TFPI levels in patients with cardiovascular disease are emerging[9,10]. Crawley and others very recently demonstrated enhanced TFPI expression in the atherosclerotic vessel[11,12]. It was thereby colocalized with tissue factor suggesting a significant role of TFPI in the regulation of tissue factor activity. They also confirmed the localization of TFPI in macrophage-derived foam cells[11]. This may implicate an important role of TFPI in counteracting the procoagulant state of these foam cells. However, an interesting finding has been reported whereby the procoagulant activity of tissue factor in foam cells, originating from monocyte-derived macrophages, appears not to be counterbalanced by an upregulation of TFPI activity[13]. This could result in a procoagulant environment at the site of the fatty streak and atherosclerotic plaque, eventually promoting cardiovascular events.

Several mechanisms play a role in the occurrence of diabetic complications such as increased oxidative stress, resulting in tissue damage[14-16]. Recently, it has been shown that oxidized LDL, as can be found in diabetic subjects, can upregulate adhesion molecules for monocytes[17]. In addition, activity of LDL-associated TFPI is impaired by lipoprotein oxidation[18,19]. This oxidative inactivation of LDL-associated TFPI may effectively neutralize its inhibitory action on tissue factor activity, compromising normal hemostasis.

Non-enzymatic glycation of proteins occurs at an accelerated rate in diabetes and can lead to the formation of advanced glycation end products (AGEs). An association of these AGEs with diabetic complications has been postulated[20]. Evidence for this association is mounting ever since[21-25]. A specific integral membrane protein has been identified as a receptor for AGE (RAGE) in endothelium, vascular smooth muscle cells and monocyte-derived macrophages[26]. This finding suggests a potential relevance of AGE-RAGE interactions for modulating properties of these tissues[27-29]. One may therefore speculate that an altered TFPI activity in diabetes could be the result of functional alterations in the endothelial cells caused by AGEs.

Is there at the moment a place in the daily clinical practice for the measurement of one or more endothelium-dependent factors ? Unfortunately, as mentioned before, up to the present only vWF appears to be of predictive value when it concerns the development of micro- and macroangiopathic complications in specific groups of diabetic subjects[2]. However, there is still a lack where it concerns the potential of an endothelium-dependent factor to predict the development of complications in the individual diabetic patient. Besides, there is also the problem of differences between specific methods for measuring an endothelium-dependent factor. TFPI can be measured with a chromogenic assay (which is expressed in % with regard to

standardized TFPI activity, measured in a plasma pool obtained from healthy donors), a clotting assay or as total TFPI antigen or free TFPI antigen with an ELISA. At this moment, there is no answer to the question which of these assays would be best to use.

One possible solution for the practical use of endothelium-dependent factors to predict complications may be the determination of cut-off points and the assessment of sensitivity, specificity, and the positive and negative predictive values of the different factors. One may even combine two or more factors in the framework of 'risk stratification' to enhance the potential of prediction. Uniformity in the determination of the levels of cut-off for the different factors is thereby of utmost importance.

In conclusion, TFPI activity is increased in diabetes mellitus, especially in subjects with type 1 diabetes and microalbuminuria. Poor metabolic control appears to increase TFPI activity. Although the results in this thesis indicate that TFPI may be a marker of endothelial dysfunction, especially in type 1 diabetes, more (prospective) studies are needed to confirm this postulation and to further elucidate its power to predict cardiovascular events. Also the exact role of TFPI in the pathogenesis of cardiovascular events warrants further investigation.

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Chapter 9

Samenvatting

Samenvatting

Tissue factor pathway inhibitor (weefselfactor stolweg remmer) in patiënten met diabetes mellitus.

Diabetes mellitus ofwel suikerziekte wordt gekenmerkt door het optreden van vroegtijdige atherosclerose, zich uitend in hart- en vaatziekten. Uit recente internationale studies zoals de DCCT (Diabetes Control and Complication Trial) en UKPDS (United Kingdom Prospective Diabetes Study) blijkt dat hoe slechter de diabetesregulatie, des te groter de kans is op de ontwikkeling van complicaties. Daarbij spelen processen als endotheelbeschadiging van de vaatwand, stoornissen in de vetstofwisseling en stollingsproblemen een belangrijke rol. Daar diabetes mellitus tot op heden niet te genezen is, is de huidige behandeling van patiënten gericht op een zo optimaal mogelijke regulatie van de bloedglucosespiegels ter voorkoming van complicaties op korte en lange termijn zoals hart- en vaatziekten (macroangiopathie), nierfunctieverlies (nefropathie), aantasting van de netvliesvaatjes van de ogen (retinopathie) en zenuwbeschadiging (neuropathie). Tot nu toe zijn er een aantal zogenaamde endotheelafhankelijke factoren (eiwitten zoals von Willebrand factor (vWF), tissue plasminogen antigen (t-PA), plasminogen activator inhibitor-1 (PAI-1) en thrombomoduline) bekend, die bij diabetespatiënten met hart- en vaatziekten verhoogd in het bloed aanwezig zijn. De gedachte is dat deze verhoogde spiegels de ontwikkeling en aanwezigheid van complicaties zoals vaatwandschade weerspiegelen. De genoemde factoren spelen alle een rol in het stollingsmechanisme (hemostase) van het bloed.

Halverwege de jaren tachtig werd een nieuw eiwit met een antistollende werking geïdentificeerd, dat essentieel bleek te zijn voor de remming van de zogenaamde extrinsieke stolweg ofwel de door weefselfactor geïnitieerde stolweg (= tissue factor pathway). Het eiwit werd derhalve aanvankelijk EPI (extrinsic pathway inhibitor), later TFPI (tissue factor pathway inhibitor) genoemd. TFPI wordt door de bekledende endotheelcellen van de vaatwand geproduceerd. Na productie wordt het eiwit grotendeels, waarschijnlijk via glycosaminoglycanen, aan de endotheelcellen gebonden, terwijl een kleiner gedeelte gebonden aan lipoproteïnen en bloedplaatjes in het bloed wordt teruggevonden. Slechts een zeer kleine fractie is ongebonden in het bloed aanwezig. TFPI kan van het vaatendotheel 'losgemaakt' worden door middel van heparine en kan als TFPI activiteit met behulp van een zogenaamde chromogene meetmethode in het bloedplasma gemeten worden. TFPI oefent zijn antistollende werking zowel in het bloedplasma als aan het oppervlak van de endotheelcellaag van de vaatwand uit.

Een van de eerste fases in het ontstaan van atherosclerose is (functionele) schade aan het endotheel van de vaatwand. Men zou daarom kunnen veronderstellen dat

TFPI een merker zou kunnen zijn voor de aanwezigheid van vaatschade. In een eerste studie met patiënten met diabetes mellitus type 1 zonder complicaties hebben wij het mogelijk effect van diabetes op TFPI onderzocht (*Hoofdstuk 3*). Allereerst bleek dat patiënten met diabetes mellitus type 1 hogere TFPI activiteit in hun plasma verbonden dan gezonde proefpersonen. Dit was zowel voor als na toediening van heparine. Bovendien werden bij patiënten, die slecht waren gereguleerd wat betreft de diabetes, een beduidend hogere TFPI activiteit in het plasma gemeten dan in patiënten met een goede metabole controle. Er bestond een duidelijk verband tussen de mate van bloedglucoseregulatie en de hoogte van TFPI activiteit, d.w.z. hoe slechter de bloedglucoseregulatie (gemeten aan de hand van het geglyceerd hemoglobine ofwel HbA_{1c}), des te hoger de TFPI activiteit in het plasma van het bloed. Uit deze gegevens lijkt TFPI activiteit een mogelijke merker van (vroege) endotheelschade te zijn.

Om dit nader te onderzoeken werd TFPI activiteit voor en na heparinetoediening gemeten in diabetespatiënten type 1 met en zonder complicaties (*Hoofdstuk 4*). Patiënten met microalbuminurie (als uiting van dreigende nefropathie) bleken zowel voor als na toediening van heparine een significant hogere TFPI activiteit te hebben in vergelijking tot patiënten zonder complicaties of met alleen retinopathie. Daar microalbuminurie algemeen beschouwd wordt als een uiting van gegeneraliseerd vaatlijden lijken de resultaten van dit onderzoek de hypothese van TFPI als merker voor vaatschade te ondersteunen.

Echter, diabetes wordt onder meer gekenmerkt door een verhoogde stollingsneiging (procoagulante status). Men zou daarom ook kunnen veronderstellen dat een verhoogde TFPI activiteit in diabetespatiënten een reactie zou kunnen zijn op deze procoagulantie. Dit werd nader bestudeerd in gezonde proefpersonen en patiënten met type 1 diabetes (*Hoofdstuk 5*). Ondanks het feit dat een verhoogde stollingsneiging in diabetespatiënten ten opzichte van gezonde vrijwilligers kunnen worden aangetoond, kon geen verband tussen deze procoagulante toestand en TFPI activiteit worden gevonden.

De toestand van een zogenaamde gestoorde glucose tolerantie (IGT) wordt beschouwd als overgangsfase in de ontwikkeling van diabetes mellitus type 2 (vroeger ook wel ouderdomsdiabetes genoemd). Het optreden van zowel IGT als diabetes mellitus neemt toe met de leeftijd. Niet alleen de leeftijd, maar ook IGT en diabetes geven aanleiding tot een verhoogde kans op het optreden van hart- en vaatziekten. In *Hoofdstuk 6* werd de TFPI activiteit in gezonde oudere personen en oudere personen met een gestoorde glucose tolerantie dan wel oudere patiënten met diabetes mellitus type 2 bestudeerd. In dezelfde populatie werd tevens vWF, t-PA, PAI-1 en thrombomoduline nader onderzocht. In de verschillende groepen konden geen verschillen in TFPI activiteit of thrombomoduline spiegels worden vastgesteld. Anderzijds werden ten opzichte van de gezonde ouderen verhoogde waarden van vWF antigeen, t-PA antigeen en PAI-1 activiteit in oudere personen met IGT en

diabetes gevonden. Wel was er een positieve relatie tussen de mate van metabole regulatie enerzijds en TFPI activiteit en vWF anderzijds. Ook de mate van eiwituitscheiding in de urine was gecorreleerd met TFPI activiteit, vWF en PAI-1 activiteit. Geconcludeerd kan worden dat niet alleen in oudere patiënten met diabetes type 2, maar ook in oudere personen met IGT reeds verschillende endotheelafhankelijke factoren zijn verhoogd, hetgeen zou kunnen wijzen op endotheelschade. Daarentegen lijkt TFPI activiteit bij ouderen minder goed bruikbaar als merker voor vaatwandschade.

Om de effecten van diabetes op TFPI activiteit nader te kunnen bestuderen werden experimenten uitgevoerd met gekweekte humane endotheelcellen, die 'onsterfelijk' waren gemaakt (*Hoofdstuk 7*). Nadat endotheelcellen in kweekmedia met verschillende concentraties glucose waren gekweekt, werden deze cellen vervolgens gedurende 10 minuten geïncubeerd met heparine. Hierna werd TFPI activiteit in het supernatant en aan het celoppervlak gemeten. Allereerst bleek hogere heparineconcentraties tot 0.6 U/ml te leiden tot toename in TFPI activiteit. Daarnaast kon worden aangetoond dat met name gedurende de eerste 10 minuten heparine het TFPI van het oppervlak van de endotheelcellaag vrijmaakte. Heparine leidde tot hogere TFPI activiteit in celkweken met hoge glucoseconcentraties, onafhankelijk van het aantal endotheelcellen en osmolariteit. Insuline toegevoegd aan de kweekmedia bleek alleen invloed te hebben op de TFPI activiteit na heparine in geval van kweekmedia met hoge glucoseconcentraties. Een lage insulineconcentratie in combinatie met een hoge glucoseconcentratie resulteerde in verhoogde TFPI activiteit, terwijl een hoge insulineconcentratie leidde tot tegengestelde effecten. Uit deze experimenten blijkt in ieder geval dat hoge glucosespiegels tot veranderde TFPI activiteit leiden.

Geconcludeerd kan worden dat zowel basale TFPI activiteit als ook na toediening van heparine is verhoogd in vivo en in vitro door chronische hyperglycemische condities. Hoewel dit niet specifiek door ons is bestudeerd, hebben wij de hypothese dat deze chronische hyperglycemie zou kunnen leiden tot veranderingen in metabolisme en/of structuur van glycosaminoglycanen, hetgeen zou kunnen resulteren in veranderde bindingskarakteristieken tussen TFPI moleculen en glycosaminoglycanen aan het celoppervlak van het vaatendotheel. Deze veranderingen zouden uiteindelijk een rol kunnen spelen bij het optreden van hart- en vaataandoeningen in diabetespatiënten.

De laatste jaren verschijnen steeds meer publicaties over verhoogde TFPI spiegels in patiënten met cardiovasculaire aandoeningen. Verschillende mechanismen zijn belangrijk voor het optreden van complicaties bij diabetes, zoals verhoogde oxidatieve stress, leidend tot weefselschade. Men heeft aangetoond dat door oxidatie TFPI, gebonden aan LDL-cholesterol, in activiteit inboet, waardoor het evenwicht in hemostase in gevaar komt, hetgeen kan resulteren in een verhoogde

stollingsneiging. Ook de vorming van zogenaamde advanced glycation end products (AGEs) wordt geassocieerd met complicaties in diabetespatiënten. Verschillende studies suggereren dat AGEs via specifieke receptoren de functies van cellen, inclusief de endotheelcellen van de vaatwand, kunnen beïnvloeden.

Is er in de dagelijkse praktijk (reeds) een plaats voor de bepaling van een of meer van de endotheelafhankelijke factoren zoals TFPI, vWF, t-PA, PAI-1 en thrombomoduline ? Tot op heden lijkt alleen vWF van voorspellende waarde te zijn waar het de ontwikkeling betreft van micro- en macrovasculaire complicaties in specifieke groepen van diabetespatiënten. Echter, ten aanzien van het vermogen van een factor om in een individuele patiënt de ontwikkeling van complicaties te voorspellen bestaat vooralsnog geen potentiële kandidaat. Eén van de problemen waartegen men aanloopt is dat er verschillende meettechnieken kunnen bestaan voor het meten van een factor. Zo kan TFPI met behulp van een chromogene methode, een stolmethode, en een ELISA gemeten worden. Vooralsnog is het niet duidelijk welke methode het beste is om te gebruiken.

Een mogelijke oplossing ten behoeve van praktische bruikbaarheid van endotheelafhankelijke factoren om complicaties te voorspellen zou de uniforme bepaling van zogenaamde cut-off points zijn, waarmee sensitiviteit, specificiteit, en positieve en negatieve predictieve waarden van de verschillende factoren vastgesteld kunnen worden. Men zou twee of meer factoren kunnen combineren in het kader van risico stratificatie om het vermogen van voorspellen te verhogen. Het vermogen de ontwikkeling van complicaties in een individu met diabetes te voorspellen kan leiden tot optimale preventieve maatregelen.

Resumerend kan gesteld worden dat TFPI activiteit is verhoogd in diabetes mellitus, met name in patiënten met diabetes mellitus type 1 en microalbuminurie. Slechte metabole controle van de diabetes blijkt TFPI activiteit te verhogen. Hoewel de resultaten beschreven in deze thesis suggereren dat TFPI een mogelijke merker van endotheelfunctie, zijn meer (prospectieve) studies nodig om dit te bevestigen. Ook de rol van TFPI bij de ontwikkeling van cardiovasculaire aandoeningen verdient verder onderzoek.

Dankwoord

Dankwoord

'Als je eenmaal specialist in een perifeer ziekenhuis wordt, kun je je promotieonderzoek verder wel vergeten' is een vaak geuite opmerking wanneer een specialist het universitaire centrum voor een perifeer centrum verruilt. Sinds ik in juli 1996 Maastricht verruilde voor Goes leek dit ook voor mij op te gaan. Al gauw merkte ik dat met de drukke werkzaamheden de grip op de tijd om het proefschrift af te schrijven steeds zwakker werd. Naarmate dit besef meer tot mij doordrong, werd besloten de zomervakanties door te brengen in vakantiehuisjes in inspirerende oorden, om alsnog het proefschrift af te ronden. Aldus werd het nuttige met het aangename met succes verenigd. Uiteraard heb ik daarbij de nodige steun ondervonden van vele personen. Zonder iemand te kort te willen doen, zou ik van de gelegenheid gebruik willen maken om aan een aantal van hen mijn dankwoord te richten.

Allereerst denk ik hierbij aan Dr BHR Wolffenbuttel. Beste Bruce, de basis van dit proefschrift lag in New Orleans, alwaar wij een congres over Lp(a) bezochten. Door de vele controverses omtrent Lp(a) was ons duidelijk geworden dat hiermee geen eer te behalen viel. Het was tijdens een etentje dat jij voor het eerst het woord TFPI liet vallen. Vanaf die tijd was het onderwerp voor toekomstig onderzoek duidelijk geworden. Voor mij ligt jouw kracht vooral in het optimaal beschikbaar maken van de randvoorwaarden, waarmee wetenschappelijk onderzoek maximaal verricht kan worden. Door altijd weer de nodige obstakels te beslechten maakte jij het voor mij mogelijk om in alle vrijheid aan het onderzoek te werken. De ongedwongenheid en het (schijnbaar) afwezig zijn van beperkingen maakten wetenschappelijke inspanningen voor mij een feest. Met plezier kijk ik echter niet alleen terug naar deze periode in Maastricht, maar ook naar onze gezamenlijke buitenlandse studiereizen. Die keer dat wij gierend van het lachen, maar badend in het angstzweet, met een kleine ATR-42 van Air Exel vanaf Beek naar Schiphol stoer windkracht 9 trotseerden zal ik nooit vergeten.

Dr K Hamulyák, beste Karly, jouw kritisch commentaar op alles wat met hemostase te maken heeft heb ik me altijd zeer aangetrokken en daarom ook enorm gewaardeerd. Daarnaast mocht ik zonder enig probleem van al jouw laboratoriumfaciliteiten gebruik maken, wat voor mij als onderzoeker een ongekende luxe betekende. Hierdoor kon met name mijn in vitro studie verder gestalte krijgen. Voor dit alles ben ik je dan ook buitengewoon erkentelijk.

Prof dr AC Nieuwenhuijzen Kruseman, beste Arie, jou ben ik zeer dankbaar voor het feit dat jij mij, bij mijn aankomst in Maastricht in 1989, het bijzondere van de Endocrinologie weer liet zien. Door mijn ervaringen als arts-assistent in opleiding op jouw afdeling heb ik met de endocrinopathologie in de volle breedte kunnen kennismaken in een sfeer die vrij uniek genoemd kan worden binnen een academische setting. Jouw stimulerende leiding in wetenschappelijk onderzoek heeft ertoe bijgedragen dat de afdeling floreerde en in korte tijd nationale en internationale bekendheid genoot op het gebied van diabetesonderzoek. Het was voor mij een genot dat ik hiervan deel mocht uitmaken. De goede relaties met Nicolaas Schaper en Jean-Pierre Sels hebben mij eveneens in positieve zin beïnvloed.

Prof John S Yudkin, I'm very grateful that you were willing to review this thesis as a member of the external review committee. Your critical remarks and considerations were well taken.

Met name wil ik René van Oerle noemen, hoofdanalist van het Laboratorium Hematologie, zonder wiens inbreng de uitwerking van dit TFPI-onderzoek nooit tot de huidige vorm zou zijn gekomen. Beste René, woorden schieten mij te kort om mijn dankbaarheid te uiten voor alles wat je met de nodige overtuiging en enthousiasme voor mij hebt gedaan. De betekenis van jouw inzet, zowel praktisch als inhoudelijk, voor dit onderzoek is voor een ieder welbekend, en kan niet vaak genoeg onderstreept worden. Mijn eerste schreden in het laboratorium geschiedde onder jouw hoede, uiteindelijk resulterend in een niet aflatende groei van onze 'turbocellen'. Al vanaf de eerste dag dat ik Maastricht had verlaten, miste ik onze levendige en soms langdurige discussies; veel plezier heb ik ook beleefd aan onze soms eindeloos lijkende fantasieën, die wij de vrije loop lieten om probleemstellingen te attaqueren. Je schuwde ook geen moment van educatie: tijdens ons hemostasecongres in Jeruzalem ervoer je aan den lijve wat de sabbat voor gelovigen in de praktijk betekende. De betekenis van een autoloze zondag was voor jou blijkbaar meer dan het gevolg van een oliecrisis. René, ik hoop dat je als paranimf deze promotie ook een beetje als jouw promotie zult beschouwen.

Dr MJ Lindhout, beste Theo, jouw kritische commentaren als biochemicus gedurende de ontwikkeling en uitvoering van met name de in vitro experimenten heb ik zeer op prijs gesteld.

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Een proefschrift kan heden ten dage slechts na een perfect redigeren naar de drukker: alleen Tiny Wouters lijkt daartoe in staat te zijn. Beste Tiny, je hebt jouw onschatbare waarde voor een promovendus wederom weten te bewijzen. Ook jouw vertrouwen in een goede afloop van dit boekje heeft mij in de loop der jaren gestimuleerd, waarvoor mijn dank.

Mam, het is duidelijk dat zonder jou dit boekje er niet zou zijn geweest. Jouw niet aflatend vertrouwen in mij is heel mijn leven tot steun geweest. Lieve Ries, in voor- en tegenspoed, ik zou niet weten welke manier van bedanken het geduld wat je de afgelopen jaren hebt moeten opbrengen recht zou doen, maar nu er weer meer tijd beschikbaar lijkt te komen kunnen we tesamen in ieder geval intenser van het leven genieten. Ons nieuw onderkomen is daartoe een goede eerste aanzet.

Curriculum Vitae

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De auteur van dit proefschrift werd op 21 januari 1958 te 's-Hertogenbosch geboren. Op het Sint Janslyceum werd het Gymnasium ß doorlopen, waarna in 1976 aan de Rijksuniversiteit Utrecht de studie Geneeskunde werd gestart. In 1984 werd het artsexamen afgelegd. In datzelfde jaar werd tevens deskundigheid Stralenbescherming nivo 3 behaald aan het JA Cohen Instituut, Interuniversitair Instituut voor Radiopathologie en Stralenbescherming te Leiden. Vanaf 1984 volgde hij de opleiding tot internist, aanvankelijk in Catharina Ziekenhuis Eindhoven (opleider Dr HFP Hillen), later in het Academisch Ziekenhuis Maastricht (opleider Prof Dr JA Flendrig). In de laatste periode van de opleiding werd tevens de opleiding in het aandachtsgebied Nefrologie (opleider Prof Dr JP van Hooff) afgerond. In 1991 vond registratie als internist-nefroloog plaats. In aansluiting hierop volgde hij de opleiding in het aandachtsgebied Endocrinologie (opleider Prof Dr AC Nieuwenhuijzen Kruseman); het was in deze periode dat een begin werd gemaakt met het huidig beschreven promotieonderzoek. Vanaf 1 juli 1996 is hij als internist-nefroloog werkzaam in het Oosterscheldeziekenhuis te Goes.